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Marine Natural Products for Protein Misfolding Modulation

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1. Introduction

Proteins are linear covalent chains of amino acids. In most proteins, the chain winds in a specific way to adopt a set fold, or "conformation", that is stable and that allows it to function. A number of proteins remain unfolded in their native state and these are referred to as intrinsically disordered proteins. These unfolded proteins remain soluble and functional and some exhibit specific ligand binding capability, which can cause them to adopt an induced fold. In contrast to these two functional situations of natively folded and disordered proteins, proteins that are normally folded or disordered can misfold, that is, to fold incorrectly.

Proteins may misfold as a result of enzymatic cleavage, post-translational modification, mutation, overabundance or structural destabilization due to alteration in the tissue environment. Accumulation of misfolded proteins can occur more readily when proteostasis mechanisms that foster correct folding of proteins, such as the chaperone system, and that clear the cell of misfolded proteins, such as the ubiquitin-proteasome system, are compromised [1]. A subset of human proteins appears to have an unusual tendency to misfold and this is recognized as a central event in a number of human diseases (reviewed by [2]). Improper folding often leads to problems because the misfolded protein cannot perform its normal role (loss of essential function) and because it assembles into oligomeric forms or larger aggregates that are toxic to the cell (gain of harmful function) [3]. The most recognized misfolded conformation is a stacking of β-sheets forming a crossed-β secondary structure that assembles into a linear multimeric fiber called amyloid (reviewed in [2]).

Several neurodegenerative diseases share protein misfolding as an underlying cause [4, 5]. These diseases can be classified based upon the proteins affected [3] while sharing a common mechanism of emergence. Protein misfolding in neurodegenerative diseases has been the focus of many reviews; excellent examples are in [5-9]. Therefore, a comprehensive re-

view will not be undertaken here. The canonical neurodegenerative diseases are Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease, amyotrophic lateral sclerosis and the prion diseases. Although each involves distinct proteins, misfolding has a common role. Huntington's disease is caused by an excessive number of sequential glutamines near the N terminus of the protein huntingtin. An expansion of the natural glutamine stretch exceeding approximately 33 residues results in Huntington's symptoms and the age of onset of the disease declines with increasing residue number [10-12]. A likely reason for this is the increased propensity for aggregation with increasing polyglutamine length [13]. Amyotrophic lateral sclerosis is a disease associated with the death of the upper and lower motor neurons in the spinal cord, brain stem and motor cortex. The affected neurons accumulate aggregate protein inclusions that may be causing the cells to die. Mutations in genes encoding superoxide dismutase-1, TAR DNA binding protein 43 and fused in sarcoma/ translation in liposarcoma (FUS/TLS) are implicated in ALS (reviewed in [14]). Prion diseases display several interesting commonalities with these other neurodegenerative diseases. Prion diseases emerge from misfolded prion protein, which serves as a template for subsequent misfolding of native prion protein with ensuing aggregation and neuron loss. The propagation of pathogenic misfolding from one protein molecule to its healthy neighbours by templating was long considered to be unique to prions; however, recent studies have suggested that molecular templating of misfolded proteins occurs with other neurodegenerative diseases as well. Furthermore, templating is inferred by observation of their spatial spread from select foci in the brain [5, 15, 16]. It is notable that prion diseases, which are infectious, may occur in animals or humans of all ages, whereas the apparently non-transmissible protein folding diseases occur mainly with ageing. This implies that a key step in all protein folding disease may be the initial establishment of a misfolded protein in a neuron, whether it is a misfolded protein taken in at any age by infection or misfolding that emerges in a susceptible endogenous protein due to faltering proteostasis with ageing. Therefore, the prevention of misfolding and/or the promotion of disaggregation and refolding of misfolded proteins *in vivo* appear to be the most direct means of dealing with all these diseases.

In this chapter, the focus is on AD and PD, as these are the two most prevalent neurodegenerative diseases, and the proteins that misfold in these diseases are well studied. AD accounts for approximately two thirds of all cases of dementia [17]; it leads to cognitive deficits in reasoning, memory, abstraction and motor skills, with eventual death. It is characterized at the molecular level by extracellular aggregates of a short peptide called amyloid beta (Aβ, and also called beta amyloid), which can form oligomers and larger fibrils with amyloid structure and later emerging intracellular neurofibrillary tangles that are composed of hyperphosphorylated tau protein (reviewed in [18]). In this chapter, Aβ normally refers to Aβ_{42}, which is the 42-residue peptide and also appears to be most toxic form. There is increasing evidence that the Aβ oligomers have a key and likely causative role in AD. Most compelling are studies showing mutations in the regulatory region or encoded protein sequence in amyloid precursor gene in families predisposed to AD [19-21]. In contrast to AD, PD has been found to involve the misfolding of α–synuclein (αS) into aggregates found in Lewy bodies and Lewy neurites. PD affects the *substantia nigra*, a region of the brain in-

volved with reward, addiction and movement. Many of the onset symptoms of PD reflect this, and the diagnosis is based on resting tremor, slowness of movement, rigidity and postural instability [22]. These two proteins are unrelated in sequence and they have distinct structures in their native form, with Aβ a short mainly unstructured peptide and αS a larger 140-residue protein that is post-translationally modified and that can exist in alternative conformations including a largely α-helical form and a disordered state [23, 24]. In spite of these differences, both proteins misfold and aggregate, and the aggregates of each can be detected and measured using assays that will be discussed in the section below.

There is growing interest in the development of protein folding modulators that would offer to prevent or alleviate misfolding and to avoid the damage that occurs in neurodegenerative diseases. There has been progress along several avenues, but no magic bullet to date. Nonetheless, there remains substantial untapped diversity among natural products and notably in marine resources. With a focus on Aβ and αS, which are implicated in AD and PD, respectively, this chapter will examine natural agents that may prevent or ameliorate protein misfolding diseases, with particular attention to the potential of marine resources for possible discovery and development.

2. Screening and evaluation for action on protein folding

With the recognition that protein misfolding is a cause of several neurodegenerative diseases and a common mechanism of their progression, promising developments have begun to emerge (reviewed in the next section). These have relied upon the measurement of protein misfolding modulation as an early step in screening and evaluation of new active molecules or as a follow-up step in the evaluation of known and promising candidates because of their suspected effects on the diseases. Identification of a molecule as a folding modulator raises the possibility that it could address the cause of AD or PD, rather than only addressing their symptoms, which makes products identified in this way potentially valuable in prevention as well as in amelioration of existing disease.

2.1. *In vitro* analysis of protein misfolding modulation

For both Aβ and αS, the transition from the native state to an amyloid conformation is not direct, but involves steps or stages. These may include unfolding, oligomerization and further aggregation into amyloid fibrils, with the possibility of off-pathway aggregation or reversal of these processes [25, 26]. This is summarized in Fig. 1. Compounds may prevent misfolding and/or templating by inhibiting the unfolding of a natively folded protein, by promoting the folding of an unfolded form, by preventing amyloid formation or by diverting the protein toward an alternative aggregation pathway leading to a less harmful aggregate. A selection of assay approaches to misfolding evaluation will be compared here in terms of information value, throughput and versatility.
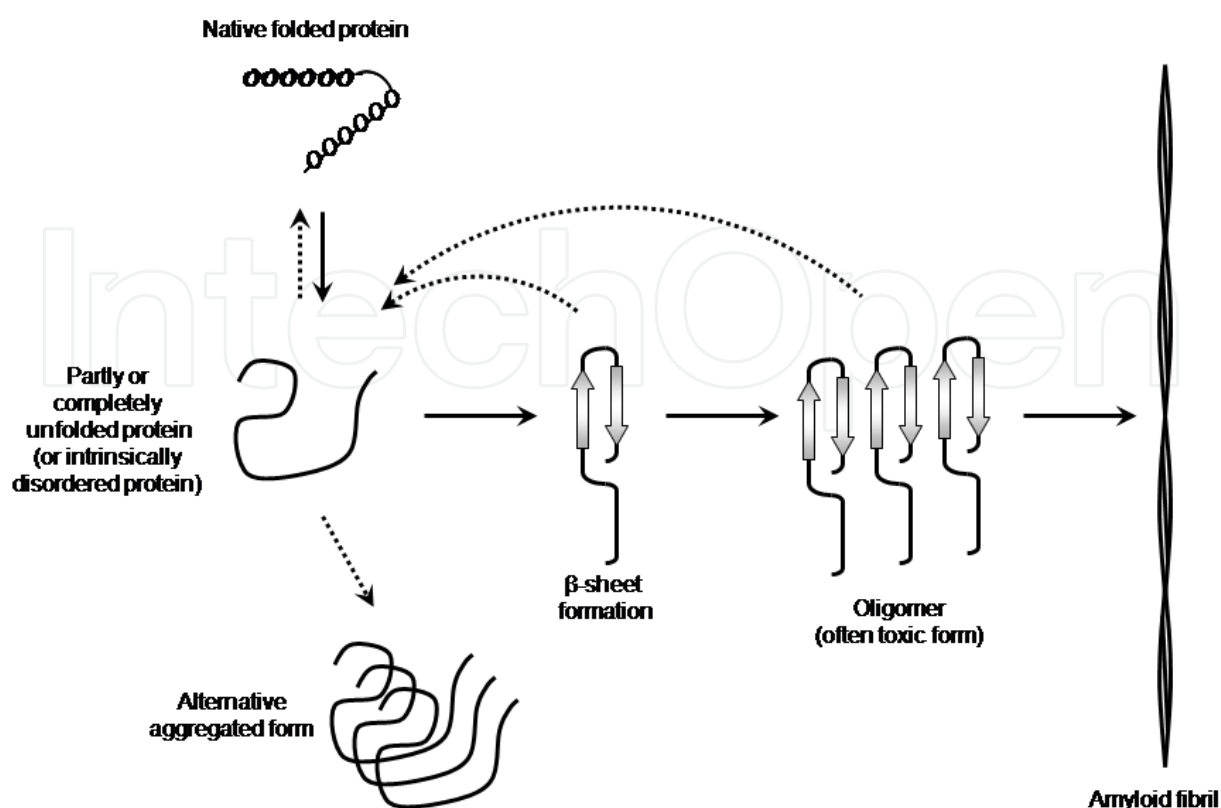
Figure 1. Overview of the general trajectory of protein misfolding with amyloid formation. Solid black arrows show pathway toward amyloid formation and stippled arrows show pathways of prevention or reversal of amyloid and related oligomers. This is an original diagram based upon information in reviews [2, 23].

2.1.1. Protein unfolding

For the discovery and development of novel agents against diseases such as AD and PD, a fuller picture of the action of a compound can be obtained by investigating the distinct steps of unfolding, aggregation, misfolding and amyloid formation. Proteins may remain largely folded and still produce amyloid or similar aggregates, provided that a local aggregation-prone sequence is exposed [27]. Nonetheless, for most proteins that readily form amyloid, it appears that that the loss of native folding is a required step [27]. Both Aβ and αS appear to have some unfolded or disordered character, but with regions that can become helical under appropriate conditions [28-30]. Inducing or preserving this helical conformation is a rational approach to preventing amyloid formation [29, 31, 32].

Folded and unfolded proteins differ in several characteristics that can be easily measured and that would allow folding modulators to be detected and evaluated. Some of the most common methods are summarized here. For example, proteins absorb right and left-circularly polarized light differently; this circular dichroism (CD) can be measured over a series of wavelengths to give informative spectra. The CD spectra of unfolded and folded proteins differ and those of proteins with predominantly α-helix and β-sheet secondary structure can be easily distinguished. In more detailed studies, the proportion of each major secondary

structure in a protein can be calculated from the spectrum using software such as DICHRO-WEB [33]. Moreover, diagnostic features such as minima at wavelengths of 208 and 222 nm for α-helical proteins can be monitored in order to quantify changes in fold in response to temperature, solution components or other factors. To date, CD has been used for small-scale studies on protein folding and protein-ligand interaction because throughput is limited to a single sample at a time. The recent development of a high-speed automated CD spectrometer (ACD, Applied Photophysics) may allow this approach to be employed more broadly in sample screening. An advantage of CD measurement is that it is an intrinsic property of a protein; thus, the spectra require no additive and the protein can be measured directly without accessory molecules or modifications. Another intrinsic feature of proteins that can be measured directly is fluorescence. Proteins with aromatic side chains fluoresce when exposed to UV light and this can undergo a shift in intensity or wavelength of intensity maximum with folding. Fluorescence can also be measured using new devices that allow miniaturized measurements in a parallel manner using a similar rapid-throughput approach (e.g. the Optim 1000, Avacta Innovative Analysis). Likewise, differential binding of dyes such as SYPRO Orange allow protein forms with different exposure of hydrophobic residues to solvent to be distinguished. In the case of SYPRO Orange, the dye is quenched when free in an aqueous solution, but it becomes unquenched when it interacts with the hydrophobic regions that are exposed upon protein unfolding and its fluorescence increases as a consequence. This measurement can be performed efficiently on large numbers of samples using devices designed for quantitative PCR [34]. Measurements by these approaches allow protein folding to be assessed and changes in fold and/or fold stability to be examined. An informative and frequently employed measure of fold stability is the melting temperature (Tm), as proteins with more stable folds normally melt at higher temperatures. The Tm can be determined by any of the above techniques. A compound that stabilizes the folded conformation of a protein would raise its Tm and this change can be easily quantified by the above approaches. A caveat with amyloid-forming proteins is that stabilized folding, as exhibited by a higher Tm, may not necessarily represent the native folding that is assumed to preclude amyloid formation. For this reason, CD is particularly valuable, as it also allows the secondary structure to be determined; a spectrum distinct from that of β-sheet would provide a reasonable indication that the stable form is native and unrelated to amyloid.

2.1.2. Amyloid-like structure

It was through dye binding that amyloid plaques were first discovered. Fibrous aggregates in tissues stained blue when treated with acid followed by iodine and the aggregates were termed amyloid because they were believed to be starch based upon the colour development (reviewed in [35]). Although it is a misnomer, the name amyloid has endured and several different dyes have become invaluable in the study of protein misfolding and amyloid formation *in vitro*.

Thioflavin T (ThT), a benzothiazole dye, and Congo red, a diazobenzidine dye, are both commonly used to quantify the amount of amyloid protein present in a solution. ThT was

first described to have amyloid-binding ability over 50 years ago [36] and it has since been in widespread use in the study of amyloidosis and the search for possible binding molecules. Congo red has also been in use for amyloid detection and quantification for several decades since its first description in that role [37]. Both of these dyes undergo a red shift in their fluorescence emission when they interact with amyloid fibrils. They cannot be used to distinguish between amyloid fibrils and smaller aggregates; however, they allow quantitative analysis and they continue to find wide use in amyloid analysis. Thioflavin S is a mixture of components derived from ThT and it has no spectral shift in absorbance or emission, which makes it suitable only for imaging and not for quantitation in solution because of high background [35]. Derivatives of N-arylaminonaphtalene including ANS and bis-ANS bind primarily to early stage aggregation in amyloid formation [38, 39]. They have not been as widely used as ThT and Congo Red, but they may find greater use with the increasing focus on aggregation pathways (see section 3 below).

Recently, there has been interest in identifying newer binding fluorophores that allow oligomeric aggregates to be distinguished from fibrils. An example is the indole compound tryptophanol. It allows specific detection and quantitation of prefibrillar oligomeric Aβ because its fluorescence is quenched only in the presence of that form of Aβ with no similar quenching in the presence of Aβ fibrils [40]. Another is the carbocyanine dye JC-1 that distinguishes different aggregation states of αS [41]. JC-1 allows real-time tracking of αS aggregation, as different fluorescent signals are emitted for monomer and fibrillar binding and the ratio of the two signals allows for real-time visualization of αS aggregation [41].

Another recent development is the elaboration of antibodies and related agents that recognize proteins exclusively in their amyloid conformation, allowing the amyloid portion of the protein in question to be detected and quantified. A monoclonal antibody raised against αS was shown to recognize the protein in its amyloid form and, although the anticipated use is mainly *in vivo*, it showed effective recognition in an *in vitro* ELISA format [42]. Novel antibodies, termed gammabodies, were engineered by grafting a series of peptide sequences from Aβ into immunoglobulin variable regions [43]. These have found use in distinguishing oligomeric and amyloid forms of Aβ and this technology would be expected to be applicable to other aggregating proteins [43]. Since an amyloid-like structure is relevant to many diseases, a new capture and detection peptoid that behaves in a manner similar to an antibody offers unusual versatility. This peptoid, based upon a 6-amino acid stretch in the human PrP sequence, binds to the amyloid forms of Aβ, αS, amylin and serpin [44].

Model proteins that undergo transition to an amyloid-like conformation may also offer alternatives for amyloid formation-related studies [45]. For example, the 37-residue natively α-helical winter flounder (*Pseudopleuronectes americanus*) antifreeze protein wflAFP-6 (HPLC-6) adopts an amyloid-like structure during the freeze-thaw process [46, 47]. This may offer an interesting model system for the study of the conversion of a protein from its native form into amyloid fibrils [47, 48]. The ability to generate rapid amyloid formation in wflAFP-6 using the freeze-thaw process would offer low background compared with Aβ assays and the consistent α-helical nature of the native AFP may also allow initial unfolding steps to be more

clearly evidenced than in proteins with less defined native structure. Nonetheless, a molecule found to be an amyloid-relevant folding modulator using AFP amyloid-like transition assays may be universal or nearly so, as in the case of the peptoid detection system described above, or it may be specific to the wflAFP-6 and have no effect on other amyloid-forming proteins. Therefore, in spite of its potential advantages, further study of the antifreeze protein model would be required before its adoption in an assay platform.

2.1.3. Protein interaction during amyloid formation

As the interaction between locally or entirely misfolded monomers appears critical to non-native protein association, there is interest in identifying molecules that directly interfere with that process. Therefore, assays for binding competition or impedance have been developed. A convenient new microplate assay employs fluorescently labelled Aβ. Unlabelled Aβ is coated onto the plate surface and then the labelled Aβ is applied in solution and allowed to bind [49]. After unbound material is washed off, the fluorescence remaining can be used to determine the level of interaction of Aβ [49]. Although the aggregation state of the Aβ in solution or on the plate is not indicated, the assay indicates the interaction between in-solution and on-plate Aβ that is inhibitable, which can be taken to represent either oligomer formation or assembly into larger fibrils. Thus, molecules that affect any part of this process can be readily identified. Nonetheless, with the aggregation state of the binding units unclear except where Aβ fragments are used, this assay does not indicate which step(s) in assembly of the monomer-oligomer-fibril are being affected.

Aβ oligomerization and fibril formation can be challenging to study *in vitro* using dyes and other methods because of spurious background oligomer formation and the difficulty in determining how much oligomer may already have formed in a starting sample [50]. For these reasons, assays that avoid these problems have garnered interest. In one example, a procedure was developed involving a semi-denaturing detergent-agarose gel electrophoresis followed by blotting and immunological detection with an appropriate antibody recognizing the protein in all its forms [51]. The method allows amyloid aggregate size distributions to be determined and the user can distinguish among the monomeric, oligomeric and large aggregated (likely fibril) forms. A drawback is that it involves a vacuum transfer of proteins from the gel to a PVDF membrane. A derived method employing capillary blotting to a nitrocellulose membrane, reminiscent of nucleic acid blotting, has made the assay more precise and scaleable for screening purposes [52]. These methods allow the association state of the starting material to be determined and the effect of any product to be evaluated in comparison. Another approach is to eliminate the possibility of aggregates in the starting solution. An example is an assay based upon the expression of the Aβ sequence in tandem with green fluorescent protein (GFP) as a fusion within a bacterial cell [53]. Aggregation of Aβ precludes GFP folding and so there is no fluorescence, whereas addition of a molecule impeding the aggregation of Aβ results in GFP folding to its native structure with consequent fluorescence. A more directly quantifiable *in vitro* form of this assay was developed using the Venus yellow fluorescent protein (vYFP) instead of GFP, which allows denaturation and refolding under precisely controlled conditions to detect molecules that promote or inhibit aggregation [50].

2.2. Cell- and organism-based analyses

Once an active extract is identified by efficient *in vitro* assay and similar assay-guided fractionation has revealed the class of molecules or the molecule that is responsible, determination of effects *in vivo* is of interest. Using culture models such as the PC12 primary neuronal cells or the SH-SY5Y neuroblastoma cell line, effects of active molecules can be determined. In a first approach, cells can be treated by transfection or other means to harbour high levels of the protein of interest and then the effect of active molecules on cell survival can be measured using one of several commercially available assays for dead cells, which measure lactate dehydrogenase leakage or other indicative parameters. However, more precise information can be gleaned by histological analysis using stains such as Congo red or thioflavin S, described above, that reveal the presence of amyloid accumulation. Immunohistochemistry using appropriate antibodies against Aβ or αS can be even more informative, as some allow distinction of oligomeric denatured forms from amyloid fibrils.

Cell-based assays can also provide an indication of the possibility of a molecule crossing the blood-brain barrier. Although Aβ circulates in the serum [54] and αS is expressed in red blood cells [55], it is considered preferable for a compound to have direct access to the brain where the pathology takes place. Therefore, blood-brain barrier models in cell culture, such as the MDR-MCCK monolayer [56], are available to predict the permeability for specific substances. Nonetheless, blood brain barrier physiology is more complex than the models or direct plasma/brain ratio determinations would predict [57]. For that reason, results must be interpreted with care.

Animal models provide critical understanding in terms of the distribution, mode of action and effectiveness of an active molecule. Besides the well-known mouse models for many neurodegenerative diseases, there are valuable invertebrate and fish models. An αS-transgenic *Drosophila* fly model is available for PD [58] and an Aβ-transgenic model for AD [59] The nematode worm, *Caenorhabditis elegans,* can be used to study AD and PD [60, 61]. Zebrafish (*Danio rerio*) have an Aβ protein precursor-encoding gene [62] and synuclein genes have been identified in this species [63]. Zebrafish may also become informative for studies of αS once the encoded proteins in this organism are better understood.

3. Protein folding modulators from common terrestrial natural products

A large number of molecules have been shown to influence the aggregation of Aβ [18] and a growing number have shown effects on αS (discussed below). Although no effective therapy for PD or AD has emerged to date, progress on a number of natural products is encouraging. This situation is not surprising, as synthetic and combinatorial chemistry normally rely on a limited number of structural scaffolds and random permutations of structures and synthesizable units [64]. In contrast, natural molecules are highly diverse and they have been subject to evolutionary pressure under distinct conditions in which valuable activities

have been selected. Therefore, they may offer a combination of variety and biological pre-selection that may make a therapeutic activity more likely [64].

3.1. Natural protein folding modulators from terrestrial sources

Several natural molecules have garnered interest because they modulate amyloid formation by Aβ (reviewed in [18, 65, 66]). A number of salient examples involving Aβ and αS are presented here.

3.1.1. Curcumin

Curcumin is a natural polyphenol found in the traditional Indian spice turmeric. This molecule has been extensively investigated, in part because of its role in traditional Ayurvedic medicine [67]. Numerous studies have shown curcumin to have wide ranging biological activities, including Aβ folding modulation. Curcumin shows promising interaction with Aβ. For example, using an oligomer-specific antibody for immunoblotting, cucurmin was found prevent Aβ oligomerization and toxicity *in vitro*. In the same study, curcumin was shown to bind to amyloid plaques in brain sections of mice transgenic for the human amyloid precursor protein [68]. In addition, mature fibrils of the slightly shorter $A\beta_{40}$ peptide were destabilized by curcumin [68]. Curcumin and other polyphenols, including tannic acid, rosmarinic acid, and myricetin, were shown to inhibit fibril formation and destabilized mature fibrils for both Aβ and αS [69]. As a result, curcumin has been a component of interest in clinical trials and, most recently the exclusive molecule in a trial for prevention of early cognitive decline and abnormal Aβ accumulation in the brain [70]. If an effect is observed, it would be interesting to see if it acts directly upon misfolding at the dosage used in subjects.

3.1.2. Epigallocatechin-3-gallate

In terms of bioactive molecules in traditional medicine, perhaps the most recognized is (-)-epigallocatechin 3-gallate (EGCG) that is found in unfermented (green) tea (*Camellia sinensis*). EGCG is a pleiotropic polyphenolic molecule with interesting protein folding modulation activity. In *C. elegans* expressing human Aβ in muscle cells, western blotting analysis using an anti-Aβ antibody showed EGCG to inhibit Aβ oligomerization [71]. Studies of Aβ and αS using ThT fluorescence, electron microscopy and other biophysical methods showed interaction with EGCG and ensuing "off-pathway" aggregation into spherical oligomers, which thereby prevented the formation of typical aggregation intermediates and amyloid fibrils by both proteins [25]. Further investigation revealed EGCG to bind directly to the β-sheet structure in the amyloid fibrils formed by Aβ and by αS and to direct their rearrangement into smaller non-toxic protein aggregates [26]. A clinical trial is planned to examine the effect of EGCG on early stage AD [72] and this may have effects based upon the protein folding modulation described here, other properties of the molecule, or a combination thereof if adequate levels are attained in the brain. Other tea compounds may offer similar protection. Theaflavins are formed by oxidation of EGCG during the fermentation process to produce black tea. These derivatives were shown to have similar activity to EGCG in modulating the polymerization of Aβ and αS [73].

3.1.3. Carotenoids and related molecules

Carotenoids (pro-vitamin A) and vitamin A are present in many commonly consumed foods. The *in vitro* examination of vitamin A and β-carotene activity on AB fibril formation, as measured using dye binding and electron microscopy suggest protective effects that appear to result from binding to a region in the C-terminal half of the peptide [74, 75]. This is consistent with reports that vitamin A and related molecules inhibit the progression of AD symptoms [76]. Also using dye binding and electron microscopy, vitamin A and β-carotene were shown to inhibit the formation of αS amyloid fibrils and to destabilize existing fibrils [77].

3.1.4. Scyllo-inositol

Another natural molecule that has garnered interest is scyllo-inositol, which is abundant in the coconut palm (*Cocos nucifera*) [78]. Scyllo-inositol is a stereoisomer of myo-inositol that stabilizes a non-toxic oligomeric aggregate of Aβ [78-80]. This is in contrast to phosphatidyli-nositol, which was found to promote fibrillogenesis and membrane insertion of Aβ [78]. Scyllo-inositol also protected against Aβ oligomer-induced inhibition of long-term potentiation [80]. A clinical trial of scyllo-inositol was inconclusive after the highest doses were discontinued [81]. Nonetheless, it remains a compound of interest provided that drawbacks can be ad-dressed.

3.1.5. Emerging sources

In addition to the well-studied molecules above, there are growing possibilities for other sources of folding modulators. For example, another source of interesting polyphenols is the wolf berry, *Lycium barbarum*, which is used in traditional Chinese medicine and was shown to protect against Aβ-related cell death [82]. Extracts obtained from other species used in traditional Chinese medicine, including cat's claw (*Uncaria rhynchophylla*) and tree peony (*Paeonia suffruticosa*) have shown relevant activity [83-85]. The tree peony contains 1,2,3,4,6-penta-O-galloyl-beta-D-glucopyranose, which prevented Aβ fiber formation and reversed the process in existing fibers [84]. In addition, other polyphenols procyanidins from apple (*Malus domestica*), silymarin from thistle (*Carduus marianus*) and reservatrol from red grapes (*Vitis sp.*) show interesting activities that may warrant further study [86-88].

3.2. Challenges with natural folding modulators

Although much attention has been brought to the natural folding modulators present in various terrestrial species, none have yet translated into meaningful therapies. The best prospect currently appears to be EGCG [89], which nonetheless presents challenges.

3.2.1. Challenges in delivery of natural products

EGCG is susceptible to decomposition during storage prior to being consumed, much of it may be destroyed by normal stomach acid during digestion, it is not highly bioavailable and it carries the possibility of hepatotoxicity (reviewed in [90]). Therefore, ideal handling, delivery and dosage would need to be determined before this molecule could be employed successfully

to manage disease-related misfolding in animals or humans. Nonetheless, studies have suggested that the use of green tea is negatively correlated to the prevalence of cognitive impairment [91] and that tea shows a dose-dependent effect in protection from PD [92]. Therefore, even with regular storage, preparation and consumption of tea as part of a normal diet, it appears that tea components, and likely EGCG or its derived theaflavins, have a measure of benefit. It is not yet clear whether the reported benefits involve the modulation of protein misfolding, and this would be an area for future study. Curcumin presents challenges similar to those of EGCG and derivatives. Dietary ingestion of curcumin appears sufficient to offer some measure of protection from neurodegenerative diseases in populations that consume it (reviewed in [93]), suggesting its bioavailability. Yet, bioavailability was found to be limiting and work has been undertaken toward improving delivery of curcumin by combining it with phosphatidyl choline, olive oil and stearic acid [94]. Similarly, plasma levels of EGCG following oral consumption were higher when the product was encapsulated in chitosan nanoparticles [95]. The promising suggestion is that formulation and delivery may be optimized to enhance availability of these protein folding modulators *in vivo*. Other compounds such as carotenoids are bioavailable and cross the blood-brain barrier as well, possibly making them convenient for the establishment of protein folding modulators [96].

3.2.2. Requirement for crossing the blood-brain barrier

It is widely accepted that molecules must cross the blood-brain barrier in order to have relevant biological effects on neurodegenerative diseases; however, interesting new findings are suggesting that this may not always be the case. In a study using radiolabeled $A\beta_{40}$ administered to the brains of rats, elevated peripheral $A\beta_{40}$ levels reduced protein clearance from the brain [97]. Conversely, a decline of $A\beta$ levels in blood plasma can lead to cognitive improvement, presumably by reducing $A\beta$ levels in the brain [98, 99]. Furthermore, genetic study of the presenilin gene encoding a secretase with a role in $A\beta$ synthesis revealed a heritable expression level in the liver, suggesting a role of liver-expressed protein in brain $A\beta$ levels [100]. Follow-up investigation using ST571, a drug that lowers peripheral $A\beta$ levels but that does not cross the blood-brain barrier, showed reduction in brain $A\beta$ [100]. Although these were studies of complete $A\beta$ levels rather than the different conformations and aggregates, together they do imply that modulation of peripheral oligomerized and fibrillar forms of $A\beta$, which would facilitate clearance, could affect the brain ratios of differently associated $A\beta$ forms. Therefore, protein folding modulators that do not cross the blood-brain barrier may still be worth considering in the prevention and treatment of disease. These studies have the drawback of assuming a stable and functional blood-brain barrier over the course of these diseases; however, other findings suggest that functioning of the blood-brain barrier may become compromised over the course of AD and other neurodegenerative diseases (reviewed in [101]), which may result in inappropriate dosages of molecules from plasma. Therefore, the prevention phase may be critical to the benefit of any molecule that acts peripherally to modulate $A\beta$, as that is the point at which normal transfer of molecules such as $A\beta$ across the barrier can occur. Alternatively, the compromised blood-brain-barrier during the disease phase may allow therapeutic molecules to reach the brain more efficiently. These possibilities require further study.

4. Marine possibilities for natural protein folding modulators

Natural products from the land include promising as sources of novel protein folding modulators for the reasons outlined earlier. By extension, marine sources may hold proportionally greater opportunity for discovery than their terrestrial counterparts because, until this decade, much of the ocean biota was unexplored. The Census of Marine Life uncovered remarkable and previously unknown plant and animal biodiversity in the oceans (reviewed in [102]). In the Gulf of Maine alone, the current species count identifies at least 652 fish species, 184 species of birds, 733 different species of microscopic plants and algae and 32 mammalian species [103]. Microbial diversity is also extensive worldwide, as inferred from the large number of new protein families uncovered from marine microbes [104].

Marine resources offer largely untapped diversity that may offer new options for natural product development [105]. Only six marine natural products and 14 synthetic compounds based upon the structures of natural marine products are FDA-approved agents or in clinical trial (reviewed in [106]). However, this list is likely to grow. In 2010, 895 new citations were reported on marine-derived compounds [107]. Marine species are already being examined for molecules that inhibit β-secretase 1, an enzyme that processes the amyloid precursor protein to give Aβ and other fragments [108]. Nonetheless, the development of inhibitors has been very difficult elsewhere [108] and there may be other biological consequences to the modulation of this enzyme. Therefore, it is ideal to focus more specifically on protein misfolding, which appears to be the linchpin in most of the neurodegenerative diseases. There is a wide variety of challenging and variable marine environments that would naturally select for traits that include protein folding modulation due to temperature or pressure, chemical defense molecules and other products that may be undersampled or undiscovered because of accessibility. Marine plants and sessile marine animals may produce chemical defenses that are remarkable in comparison to those of species that can move away from danger. In addition, species that inhabit highly variable or extreme environments, such as freezing waters, have adaptations to maintain proteostasis during exposure to extreme temperatures and solution properties [109-111]. For these reasons, they may represent ideal sources of natural molecules to prevent or ameliorate protein misfolding.
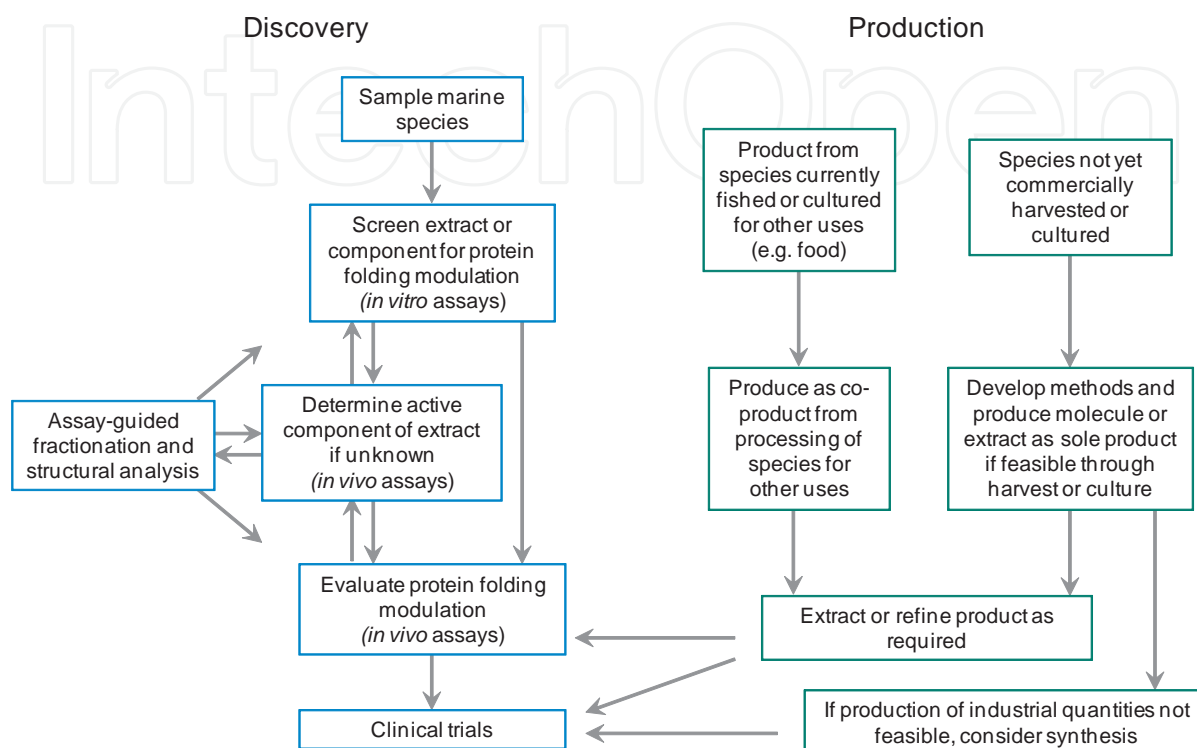
The discovery, characterization and production of marine-derived molecules appears promising, but with complexity and risk, nonetheless. A general overview of the process is shown in Fig. 2.

4.1. Natural protein folding modulators from marine sources

4.1.1. Marine polyphenols

Polyphenols are abundant and varied in marine algal species (reviewed in [112]). Like their terrestrial counterparts, algal polyphenols have shown folding modulation of Aβ. The brown seaweed (macroalgae) *Ecklonia cava* is found in the waters around Japan and Korea, where it is used as a herbal remedy. A butanol extract from *E. cava* has been shown to prevent production and aggregation of Aβ and to reduce amyloid plaques [113]. Electron microscopy showed

the Aβ oligomers to be reduced and dye binding as well, indicating an inhibition of fibril formation. The polyphenolic phlorotannins are considered to be the active compounds responsible for the biological activities of *E. cava* [114, 115]. Nonetheless, other molecules cannot be strictly ruled out [113].

Figure 2. Schematic summary of key steps in the identification and development of marine-sourced protein folding modulators. Blue boxes are steps involved in discovery and characterization of protein folding modulation and the molecule responsible. Green boxes are steps involved in production of the molecule (or of an extract containing it). For more detail, see chapter text. Structural elucidation is not included here as it is not covered in this chapter; it is covered in another chapter of this book.

4.1.2. Marine carotenoids

Marine algae and invertebrates harbour large quantities of carotenoids. This is of interest because, as noted above, carotenoids are among the molecules showing promise in modulation of protein misfolding. Algal carotenoids are widely varied (reviewed in [112]). These appear to be particularly accessible through enzyme-assisted extraction (reviewed in [116]) and they may have interesting activities. Shrimp and crab processing wastes are also excellent sources of carotenoids. Crustacean shells left over during processing contain the carotenoid astaxanthin (reviewed in [112]), which gives them their striking colour. This is in addition to the chitin in the shells, and its component glucosamine, which is well known in other contexts. There are no reports of evaluation of marine-derived carotenoids in protein folding modulation, as there are for terrestrial counterparts. With the numerous and varied sources of marine carotenoids, these could be compared with terrestrial versions previously reported to modulate protein folding.

4.1.3. Marine toxins

There is a plethora of marine toxins with distinctive neurological effects. These toxins are mainly known for the poisoning risk they carry. Nonetheless, a few of them have valuable biological activities as research chemicals and one has intriguing effects relative to AD. Using cell-based assays, the toxin 13-desmethyl spirolide C from the dinoflagellate *Alexandrium ostenfeldii* was shown to reduce Aβ accumulation in cells by just over 40% [117]. The antibody used (6E10, Covance) detects Aβ without distinguishing between monomeric or oligomeric or fibril forms. It would therefore be valuable to repeat these analyses using fold-specific anti-Aβ antibodies or another fold-sensitive method to see if any effect there could be detected. Otherwise, it may act on Aβ in other beneficial ways that would also be of interest.

4.1.4. Marine-sourced chemical chaperones and conflicting results

A variety of chemical chaperones, which are loosely defined as small molecules that promote folding of many proteins, have gained interest in terms of wide-spectrum protection of proteins from misfolding (reviewed in [118]). In marine species faced with protein misfolding risk, the accumulation of one or more chemical chaperones is a common adaptation because these small chaperoning molecules promote general proteostasis [111, 119]. Therefore, at first glance, these molecules would appear to be ideal in terms of proteins such as Aβ and αS. They could be produced naturally or synthetically, depending upon their molecular features, and they may stabilize many different proteins. However, some chemical chaperones may have problematic effects with respect to amyloidotic protein misfolding. Glycerol at supraphysiological concentrations (molar range) and trimethylamine oxide (TMAO) at moderate concentrations were both shown to favour the transition of Aβ from its unfolded conformation to the β-sheet form requisite for fiber formation [120]. Protofibril to fibril conversion was also enhanced [120]. The situation appears more complex for αS, with elevated concentrations (molar range) of TMAO favouring a partially folded form with high propensity for fibril formation and even higher concentrations of TMAO favouring an oligomeric α-helical conformation [121], which may be consistent with a native α-helical form of the protein that resists misfolding [29]. Although these chaperone concentrations used were far in excess of those that would be reached *in vivo*, the effects suggest a possibility to be aware of if a novel folding modulator appears to be non-specific. Stabilization by chemical chaperones may bring an increased risk of aggregation for some proteins [119]. Furthermore, stabilization of a wider range of cell proteins or protein complexes may cause unanticipated problems. Therefore, examination of unusual and off-target effects would be prudent in the evaluation of chemical chaperones.

4.2. Sources for marine products

A challenge presented by many rare or remote marine species that may produce pharmaceutical or nutraceutical molecules is the difficulty in obtaining sufficient product in a sustainable manner, from both the environmental and economic standpoints [122]. Options for these products include the development of culture methods for the species or synthesis of the molecule, although in many cases the molecules are too complex to be efficiently synthesized [122, 123]. In contrast, an advantage for nutraceutical or drug development from many

accessible marine species, both plant and animal, is that they are already supplied as human food sources. Additionally, a large number of marine species are currently farmed, and availability of a standard supply for natural product development would be more straightforward and predictable than it would be for their wild counterparts. Sustainable production or harvesting of species for natural marine products would be a key consideration in production of folding modulators, just as it is for the production of foods.

In the case of some seaweeds, marine harvesting can be carried out. However, on-land cultivation offers the additional benefits of ideal traceability and process control, albeit with substantial costs [124]. For microalgae, this would be the only option. Algal processing wastes are significant and they are of interest for the development of by-products [125].

For commercially fished animal species, processing for food production is a frequent practice. These wild finfish and shellfish species, as well as those grown in aquaculture, can generate substantial waste during processing for food production and this waste often go to landfills or composting. The identification of valuable properties or activities in waste materials allows them to be used to produce co-products and, with green chemistry initiatives, these could be produced in a sustainable fashion from a healthy wild harvesting or aquaculture operation. There is growing interest in co-product development, as highlighted by two contrasting examples. Snow crab (*Chionoecetes opilio*) are fished across the Maritime provinces of Canada. Crab processing waste currently contributes to landfills, but the potential for additional product development with the identification of sufficiently valuable uses is being considered [126] as is the case elsewhere, likely based upon chitin but possibly upon other products as well. Innovative solutions to waste challenges are also being pursued in salmon aquaculture. Integrated multi-trophic aquaculture of Atlantic salmon (*Salmo salar*) with macroalgal species and mussels (*Mytilus edulis*) in the Bay of Fundy, Canada, minimizes waste accumulation by allowing nutrient and resource recovery with diminished impact on the local environment and this process is advancing toward commercial production [127]. The economic viability of this integration initiative will rely upon the development of foods along with other high-value products from the cultured species [128]. A neuroprotective protein folding modulator from such a resource could enhance markets for the food, while offering the possibility of producing sustainable nutraceuticals and eventual pharmaceuticals as co-products if appropriate.

5. Conclusions

With protein misfolding identified as a causative event in multiple neurodegenerative diseases and with no definitive means of prevention or cure in place, the progress being made in the discovery and development of natural protein misfolding modulators is of great interest. Although several promising leads from terrestrial sources have been identified, none have been shown to provide a clear result in disease outcome to date. Therefore, while current products are being developed and refined, it would be logical to investigate a wider range of sources for protein folding modulators. In this context, marine species deserve a second (or, in some cases, a first) look.

There has already been extensive work on marine molecules from some sources, but crucial to the proposed endeavour is screening for the most relevant activities. For example, testing marine molecules or extracts for effects in model Aβ or αS cell survival assays may not indicate protein folding modulation. However, the reverse is likely to be true in most cases. In other words, appropriate protein folding modulation activity should lead to enhanced cell survival. It is also important to be mindful of assay subtleties. Testing for anti-fibrillization activity may uncover molecules that cause toxic oligomers to accumulate instead of amyloid fibrils, leading to increased pathology instead of amelioration. Similarly, searching for molecules that bring about a reduction in total Aβ (or αS) may not be prudent, as we have little understanding of the normal roles of these proteins in the brain and therefore drastic reduction in levels may have undesirable consequences [18]. For these reasons, an assay suite that allows determination of toxic oligomers as well as other (non-toxic) oligmers, monomers, amyloid fibrils and other aggregates would be ideal. In order to identify a marine extract or molecule that provides protective protein folding modulation, the spectrum of its effects on protein misfolding and association protein would need to be investigated. Furthermore, because a number of natural molecules appear to affect the misfolding trajectories of both Aβ and αS, these would be of particular interest because of the potential for wider-ranging protection from toxic misfolding.

In closing, examination of marine biota for protein folding modulators using a suitable suite of assays may offer a promising opportunity to identify a safe, effective and sustainable protein folding modulator that addresses the cause of a neurodegenerative disease. The untapped diversity of marine species combined with a rational selection of assays for folding modulator identification based upon examples used in terrestrial investigations and coupled with appropriate downstream *in vivo* analysis would be ideal. For proteins such as Aβ and αS, which form similar amyloids and can even cross-seed in spite of their structural differences [129], a further tantalizing prospect would be the identification of a molecule that would be effective for both AD and PD. Given the paucity of treatments currently available, discovery of an active molecule from a marine resource could bring hope at this time.

Acknowledgements

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