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vii. Abstract and keywords

The production, quality control, and degradation of proteins is a tightly controlled process necessary for cell health. In order to regulate this process cells rely upon a network of molecular chaperone proteins that bind misfolded proteins and help them fold correctly. In addition, some molecular chaperones can target terminally misfolded proteins for degradation. Neurons are particularly dependent upon this 'proteostasis' system, failures in which lead to neurodegenerative disease. In this review we identify opportunities for modulating molecular chaperone activity with small molecules, which could lower the burden of misfolded protein within neurons, reducing cell death and ameliorating the effects of neurodegeneration.

Abbreviations:

CCT	chaperonin containing TCP-1
CHIP	carboxy terminus of Hsp70-interacting protein
CMA	chaperone mediated autophagy
Hip	HSP70 interacting protein

HOP	Hsp70-Hsp90 organizing protein
HSP	heat shock protein
PAINS	Pan-Assay Interference Compounds
sHSPs	small heat shock proteins
UPS	ubiquitin proteasome system

viii. Main text

The proteostasis network

Protein structure results from the folding of a polypeptide chain, in a process that is energetically favourable and determined by the primary peptide sequence (Anfinsen, 1973). Folding is driven by the hydrophobic effect, which causes hydrophobic stretches of peptide to bind together in the centre of a protein, avoiding interaction with solution (Charton & Charton, 1982; Rose, Geselowitz, Lesser, Lee & Zehfus, 1985). Further conformational changes are driven by hydrogen bonding, salt bridge formation, and Van der Waals forces resulting in natively folded protein. However, the path to folding is not a smooth one; proteins encounter false local minima in the 'rough energy landscape' in which they fold (Dill & Chan, 1997). Often this is caused by inappropriate hydrophobic interactions within the protein, which are energetically unfavourable to reverse, trapping the protein

in a misfolded state (Rothwarf & Scheraga, 1996). Consequences of misfolding include loss of function and aggregation – a key feature in neurodegenerative diseases such as Alzheimer's, Parkinson's and Huntington's (Schulz & Dichgans, 1999). To prevent misfolding a class of proteins known as molecular chaperones assist protein folding and prevent aggregation (Figure 1). Molecular chaperones have hydrophobic binding surfaces, which bind to exposed hydrophobic regions of peptides preventing inappropriate inter- or intra-molecular interactions (Hendrick & Hartl, 1995). Molecular chaperones can also break apart aberrant hydrophobic interactions to re-fold misfolded protein or re-solubilise aggregates (Skowyra, Georgopoulos & Zylicz, 1990). If a protein is terminally misfolded, molecular chaperones can target it for degradation, either through the ubiquitin proteasome system (UPS), or by chaperone mediated autophagy (CMA) (Connell et al., 2001; Kaushik & Cuervo, 2018).

Molecular chaperones function is critical for cell health, with post-mitotic cells such as neurons particularly vulnerable to failures in chaperone activity. This review will focus on four major chaperone families; the heat shock protein 70 family (HSP70s), HSP90s, Chaperonins, and small heat shock proteins (sHSPs) (Figure 2). Chemical modulators of targets of interest are described throughout. For brevity the focus is limited to selected small molecule tools. It should be noted that beyond N-terminal ATP competitive HSP90 inhibitors, the field suffers from a dearth of well characterised compounds for probing biological function or to serve as 'pathfinder' molecules for validating functional relevance in neurodegenerative disease models or as starting points for medicinal chemistry programmes. Potential limitations of the select tool compounds are highlighted in Table 1**,** where - in the authors' opinion - a compound lacks sufficient published evidence to be considered a 'fit-for-purpose' tool or contains a sub-structure of known **P**an-**A**ssay **In**terference Compounds (PAINS). For further insight into what constitutes a 'fit-for-purpose' chemical tool,

readers are directed to an excellent recent perspective by Blagg and Workman (Blagg & Workman, 2017).

HSP70, CHIP, HOP and HIP

The HSP70 chaperone family contains at least 13 gene products expressed in humans, which differ in sub cellular location, tissue distribution, and responsiveness to stress conditions (Radons, 2016). These proteins bind to misfolded protein with a substrate binding domain, consisting of a β-sheet containing a hydrophobic groove, and an α-helical lid. Closure of the lid is caused by ATP hydrolysis in the adjacent nucleotide binding domain, which leads to tight substrate binding (Zhu et al., 1996). In isolation HSP70 has low ATPase activity and low turnover of substrate, however this activity can be catalysed to biologically relevant levels by the HSP40 family of co-chaperone proteins (Horne, Li, Genevaux, Georgopoulos & Landry, 2010; Misselwitz, Staeck & Rapoport, 1998) which also confer substrate specificity upon HSP70. The HSP40 family has over 50 members in humans with a high degree of structural diversity (Kampinga & Craig, 2010). This allows them to bind specific misfolded client proteins prior to HSP70/HSP40 complex formation. Upregulation of certain HSP40s may therefore be a viable strategy to treat neurodegenerative disease; mutations in these proteins can lead to neurodegeneration (Zarouchlioti, Parfitt, Li, Gittings & Cheetham, 2018) and HSP40 family members of particular interest include DNAJB2, DNAJB6 and DNAJB9 which bind to polyQ containing proteins, α-synuclein and β-amyloid respectively. There is a paucity of small molecules reported to modulate HSP40 activity. One compound of interest, 115-7C **[1]**, described by Wisén and colleagues (Wisen et al., 2010) has been shown to act as an 'artificial HSP40 like co-chaperone' that stimulated prokaryotic HSP70 (DnaK) ATPase and protein-folding activities. Additionally, the authors

demonstrated that treatment with **[1]** led to a reduction in protein aggregate size in a yeast polyQ aggregation model system.

The ATPase activity of HSP70 is further modulated by HSP70 interacting protein (Hip) which delays the release of ADP from HSP70, delaying substrate release. This has been shown to inhibit aggregation of misfolding substrates (Howarth, Glover & Uney, 2009; Roodveldt et al., 2009). Promoting this activity could have beneficial effects in neurodegeneration and compounds have been identified based on the rhodocyanine motif, such as pyridinum salts MKT-077 **[2]** and YM-01 **[3]**, which have been proposed to bind an allosteric site on HSP70, delay release of ADP and reduce polyQ androgen receptor levels in cell and *Drosophila* experiments (Wang et al., 2013). Neutral derivative YM-08 **[4]** has been shown to be blood-brain- barrier permeable in a mouse PK study, as well as reducing total and phospho-tau in brain slice cultures from transgenic mice expressing mutant tau (Miyata et al., 2013).

Once a protein has been released by the HSP70/HSP40 complex it can resume attempts at protein folding. Some proteins will misfold more than once, requiring multiple rounds of HSP70 binding and release. For these proteins a 'triage' decision is required – should further energy be invested in attempting to fold the protein correctly, or should it be degraded? This is decided by competitive binding of two proteins, carboxy terminus of Hsp70-interacting protein (CHIP) and Hsp70-Hsp90 organizing protein (HOP), to the EEVD motif in the C-terminus of HSP70 (Kundrat & Regan, 2010). CHIP is an E3 ubiquitin ligase which targets terminally misfolded proteins for degradation by the UPS (Murata, Minami, Minami, Chiba & Tanaka, 2001). In contrast HOP mediates the transfer of client peptide from HSP70 to another chaperone system, HSP90, which specialises in peptides with more complex folding requirements (Johnson, Schumacher, Ross & Toft, 1998). In the case of

pathologically aggregating proteins such as tau and α-synuclein it could be beneficial to upregulate CHIP binding or inhibit HOP binding to promote degradation over further refolding attempts. Using AlphaScreen Technology, Yi and Regan reported a series of Toxoflavin derivatives as inhibitors of the HSP90 – HOP protein – protein interaction (Yi & Regan, 2008).

In spite of the careful efforts by the authors to exclude false positives, it should be noted that Toxoflavin scaffolds such as **[5]** and **[6]** have been shown to be redox cycling compounds and are considered PAINS (Johnston, 2011).

Other potential targets include inhibiting the methyltransferase METTL21A which methylates K561 of HSP70) or inhibiting the phosphorylation of the C-terminus of HSP70, both of which approaches would promote CHIP binding (Jakobsson et al., 2013; Muller et al., 2013)

HSP90 and co-chaperones

Human HSP90 family members include GRP94, in the endoplasmic reticulum and TRAP1 in the mitochondria. Cytosolic HSP90s are differentiated by their response to stress – HSP90-α is induced by stress such as heat shock, whereas HSP90-β is constitutively expressed (Schopf, Biebl & Buchner, 2017).

HSP90 is a homodimer, with a C-terminal 'TPR' domain containing a 'MEEVD' motif for interactions with HOP and other binding partners, a middle domain which binds client protein and an ATP binding domain in the N-terminus. Closure of the dimer is driven by ATP binding, with ATP hydrolysis triggering dimer opening and client protein release (Maruya, Sameshima, Nemoto & Yahara, 1999; Prodromou et al., 2000; Schopf, Biebl & Buchner, 2017).

The inhibition of HSP90 has been proposed as a therapeutic route in neurodegeneration, as reduction in HSP90 activity leads to increased HSP70 levels, mediated by the transcription factor HSF1 (Luo, Sun, Taldone, Rodina & Chiosis, 2010). Higher HSP70 levels enhances the degradation of Tau and poly-Q containing proteins, which contribute to neurodegeneration (Luo, Sun, Taldone, Rodina & Chiosis, 2010). Furthermore, HSP90 client proteins include alpha-synuclein and tau (Luo, Sun, Taldone, Rodina & Chiosis, 2010), and it has been hypothesised that by stabilising their aggregation prone intermediates HSP90 contributes to their aggregation (Luo et al., 2007). However, systemic HSP90 inhibition is toxic (Blair, Sabbagh & Dickey, 2014), and HSP90 inhibitor development has thus far been driven by chemotherapy drug development as tumour cells require large amounts of HSP90 activity. The inhibitors delivered by this development are ATP competitive inhibitors at the N-terminus, and in the case of the brain penetrant inhibitor HSP990 (not shown), actually caused neurotoxicity (Spreafico et al., 2015).

Whilst systemic HSP90 inhibition is being investigated for neurodegeneration more specific strategies may have better substrate specificity and lower neurotoxicity. One approach is to target the cytosolic HSP90α isoform alone whilst another is to target co-chaperones of HSP90.

Over 20 different co-chaperones of HSP90 have been discovered to date with a variety of functions including ATP hydrolysis modulation and effects upon client protein such as proline isomerisation (Taipale et al., 2014). Examples of targets for neurodegeneration include Aha1 which stimulates HSP90 activity by promoting ATP hydrolysis, and FKBP51 which isomerases HSP90 substrates including tau at proline residues (Blair, Sabbagh & Dickey, 2014). Inhibition or knockdown of both of these HSP90 cofactors has been shown to reduce levels of tau aggregation, with effects which may be more specific and less toxic than the effects of HSP90 inhibition (Blair et al., 2013; Shelton, Koren

& Blair, 2017). A number of small molecules have been reported to abrogate the Aha1–HSP90 interaction; KU-177 **[7],** a truncated derivative of the natural product noviobiocin, a C-terminal domain inhibitor of HSP90, was reported to reduce tau aggregation through inhibition of the Aha1–HSP90 protein-protein interaction (Shelton, Koren & Blair, 2017). Stiegler et al reported Ham-1 **[8]** - an apparent allosteric inhibitor of HSP90 – that abolished Aha1 mediated stimulation of HSP90 ATPase activity but did not significantly decrease native HSP90 ATPase activity nor dissociate the protein complex (Stiegler et al., 2017). Ihrig and Obermann conducted a screen for inhibitors of the Aha1–HSP90 interaction using AlphaScreen Technology and identified A12 **[9]** and A16 **[10]** as hit compounds but did not demonstrate which protein partner the compounds primarily interacted with (Ihrig & Obermann, 2017). Gaali et al, have reported a series of potent ligands for FKBP51, exemplified by IFit4 [**11**] that are exquisitely selective over the closely related family member FKBP52 due to an induced fit binding mode (Gaali et al., 2014).

Chaperonins

Approximately 10 % of cellular protein is diverted from HSP70 by prefoldin proteins to TRiC, a cytoplasmic member of the 'chaperonin' family of proteins. TRiC has two octomeric subunit rings, comprised of chaperonin containing TCP-1 1 (CCT1) to CCT8, which enclose a central cavity shielding client protein from other proteins and reducing the entropic cost of long range intra-protein interactions. The closure and opening of a lid to this cavity is mediated by ATP binding and hydrolysis (Lopez, Dalton & Frydman, 2015).

TRiC clients include mHtt protein found in Huntington's disease (Kitamura et al., 2006). TRiC encapsulates misfolded mHtt oligomers, preventing propagation into larger fibrils. A mHtt fibril too

large to be encapsulated binds to the external face of subunits CCT1 and CCT4, 'capping' the end of the fibril inhibiting further elongation (Shahmoradian et al., 2013).

Upregulating TRiC activity may be possible by modulating its regulation. TRiC is targeted for degradation by ubiquitination at subunits CCT1 and CCT4 (Kim et al., 2014). The deubiquitinase USP25 prevents this, but is in turn inhibited by a kinase, VRK2. Small molecule inhibitors of VRK2 may therefore increase TRiC levels, reducing aggregation of proteins such as mtHtt (Kim, Lee, Lee, Song, Kim & Kim, 2015).

sHSPs

Humans have 11 sHSPs, HspB1-11, all of which are 42 kDa or smaller. sHSPs act as 'holdases', preventing protein aggregation by binding to hydrophobic patches on misfolded protein with a conserved 100 amino acid α-crystallin domain. Heat stress triggers conformational changes in sHSPs which expose the α-crystallin domain and allow sHSPs to form ordered oligomers. These activities buffer protein misfolding, preventing the formation of disordered aggregates until the stress-causing event has passed. sHSPs are ATP-independent and there are no apparent strategies to specifically upregulate their activity, but these proteins are critical as part of the wider response to protein misfolding and could respond to strategies which upregulate the global chaperone system.

Upregulation of the global chaperone system

As an alternative to modulating individual components of the proteostasis network an upregulation of the heat shock response may ameliorate neurodegeneration. Efforts in this area have focused on HSF1 – the master transcription factor of the proteostasis network (Neef, Jaeger & Thiele, 2011).

However, as HSF1 is responsive to proteotoxic stress, much of the chemical matter reported to activate HSF1 does so through non-specific cell-stress mechanisms, exemplified by molecules containing reactive functional groups that are unlikely to be of general utility. To avoid this issue a humanised yeast screen for activators of human HSF1 that do not cause proteotoxic stress was developed and identified a small molecule, HSF1A **[12]** (Neef, Turski & Thiele, 2010). This molecule activates HSF1-mediatiated chaperone protein expression via inhibition of TRiC in a non-ATP competitive manner (Neef, Jaeger, Gomez-Pastor, Willmund, Frydman & Thiele, 2014), elevating HSP70 protein levels in various mammalian cell and fruit fly models of polyQ protein aggregation. Another promising molecule is the carboximidoyl chloride derivative arimoclomol [**13**] (Vigh et al., 1997), a co-inducer of the heat shock response that prolongs stress-induced activation of HSF1 without promoting HSF1-dependent chaperone expression alone. Although the mechanism of action for arimoclomol **[13]** remains unclear it has entered clinical trials, being well tolerated in Phase I and currently in Phase II/III for superoxide dismutase 1 positive ALS (NCT00706147).

Conclusion

The proteostasis network plays a critical role in preventing neurodegeneration by ameliorating protein misfolding and targeting misfolded protein for degradation. Here we have indicated several possible therapeutic avenues which could be targeted with small molecules. It is heartening to see progress already being made in areas such as the HSP90 co-chaperones Aha1 and FKBP51, and the upregulation of the heat shock response. An improvement in the quality of tool compounds would greatly benefit further investigation into this protein homeostasis system and its role in neurodegeneration.

Nomenclature of Targets and Ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding et al., 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander et al., 2017).

Competing interests:

T. Newton is working with Reflection Therapeutics

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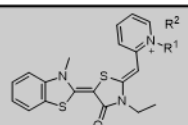
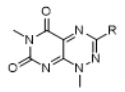
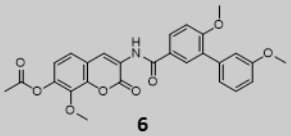
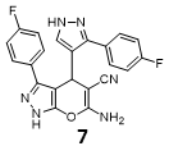
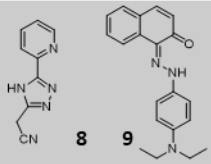
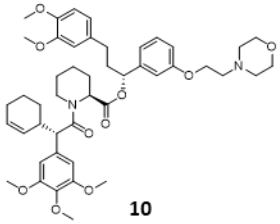
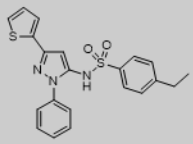
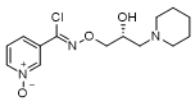
x. Tables (each table complete with title and footnotes)

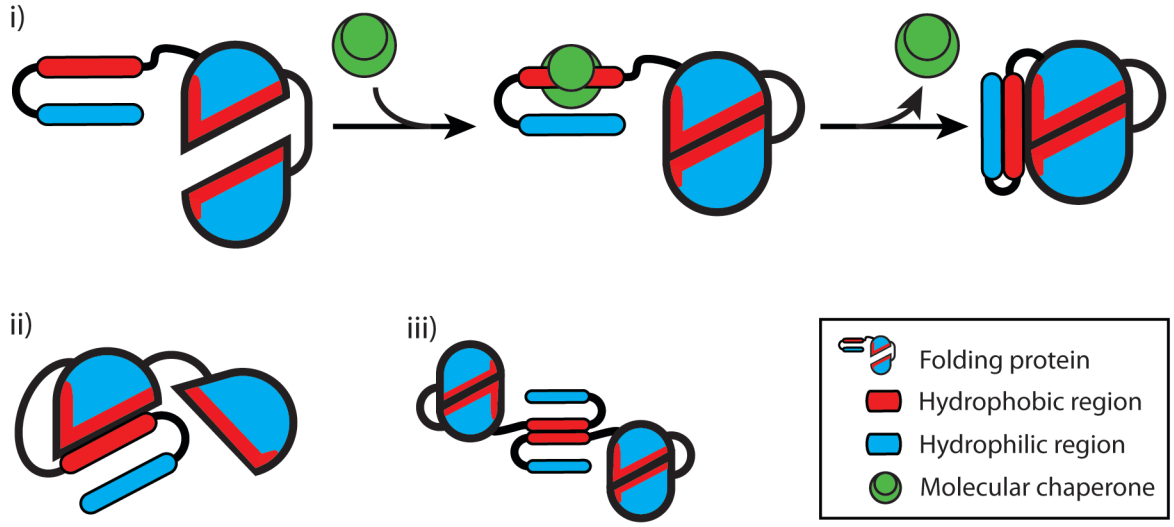
Table 1: Compounds reported to modulate the proteostasis network

Chemical Structure	Non-IUPAC Name	Reported Mechanism of Action	Potential Limitations
 **1–3**	**1**: MKT-077 – R^1 = Et, R^2 = Cl^- ; **2**: YM-01 – R^1 = Me, R^2 = Cl^- ; **3**: YM-08 – Unsubstituted	Allosteric inhibitors of HSP70 that reduce tau and polyQ androgen receptor levels in cell and animal models	Rhodocyanines known PAINs motif. 1-3 all contain reactive electrophiles (Michael acceptors). 1 and 2 display nephrotoxicity and are non-brain penetrant
 **4–5**	**4** R = Me **5** C9 R = n-Pr	Bind to TPRA2A domain of HOP inhibiting HOP–HSP90 interaction. Cytotoxic to various breast cancer cell lines	No reported evidence of efficacy in models of neurodegeneration. Toxoflavin skeleton known PAIN motif through redox cycling mechanism
 **6**	**6** = KU-177	Inhibition of Aha1–HSP90 binding interaction. Proposed to be through binding to Aha1. Reduced levels of insoluble tauP301L in cells	7-OAc group on coumarin ring likely to be labile; 2-chromenone skeleton is a potential electrophile (Michael acceptor). Requires further validation in vivo
 **7**	**7** = Ham-1	Inhibition of Aha1 mediated activation of HSP90 ATPase activity by binding allosteric site adjacent to N-terminal domain nucleotide binding site of HSP90	No reported evidence of efficacy in models of neurodegeneration. Ham-1 contains a potentially reactive nitrile and is reported as a mixture of atropisomers
 **8** **9**	**8** = A12; **9** = A16	Both A12/A16 were reported as hits for inhibition of the Aha1–HSP90 interaction using Alphascreen. Nature of inhibition unknown	No reported evidence of efficacy in models of neurodegeneration. A16 contains multiple PAIN substructures
 **10**	**10** = IFit4 More potent analogues from primary fluorescent polarization assay include SAFit1 and SAFit 2 (not shown)	Inhibition of PPIase domain of FKBP51. X-ray structure of iFit4 bound to PPIase domain of human FKBP51 is available in the PDB (ID: 4TW7)	No reported evidence of efficacy in models of neurodegeneration. High molecular weight, predicted logP and topological polar surface area are likely to limit blood-brain barrier penetration. Further in vivo evaluation required
 **11**	**11** = HSF1A	Activation of HSF1 by blockage of the inhibitory interaction between the chaperonin TRiC and HSF1. HSF1A shown to upregulate HSP70 in mammalian cell and fruit fly models of polyQ protein diseases	No obvious chemical liabilities. Reported cellular efficacy only at high concentration (>10 μM). Further studies describing the physicochemical properties of HSF1A along with in vivo validation in a mammalian model is desirable
 **12**	**12** = Arimoclomol (citrate salt)	Mechanism of action unknown. Hypothesized to prolong the time transcription factor HSF1 binds to heat shock response element	Limited published data on physicochemical properties. Reported to have a short in vivo half life – further studies would be useful to better understand PK-PD relationships in disease models. However, well tolerated in Phase I clinical trials and currently in Phase II/III trials for SOD1-positive ALS

xi. Figure legends

Figure 1: Protein folding is aided by molecular chaperones. Exposed regions of hydrophobic peptide (red) are transiently bound by molecular chaperones (green), shielding hydrophobic regions from contact with solution and allowing correct protein folding to take place (i). In the absence of molecular chaperones hydrophobic regions will avoid solution by forming inappropriate intra- (ii) and inter- (iii) molecular interactions.

Figure 2: Sites of small molecule intervention in the proteostasis network. Chaperone systems are indicated by blue circles, co-chaperones by orange ovals and protein folding pathways by arrows. Tool compounds which are reported to modulate selected targets within the proteostasis network are numbered, with more detail provided in Table 1.



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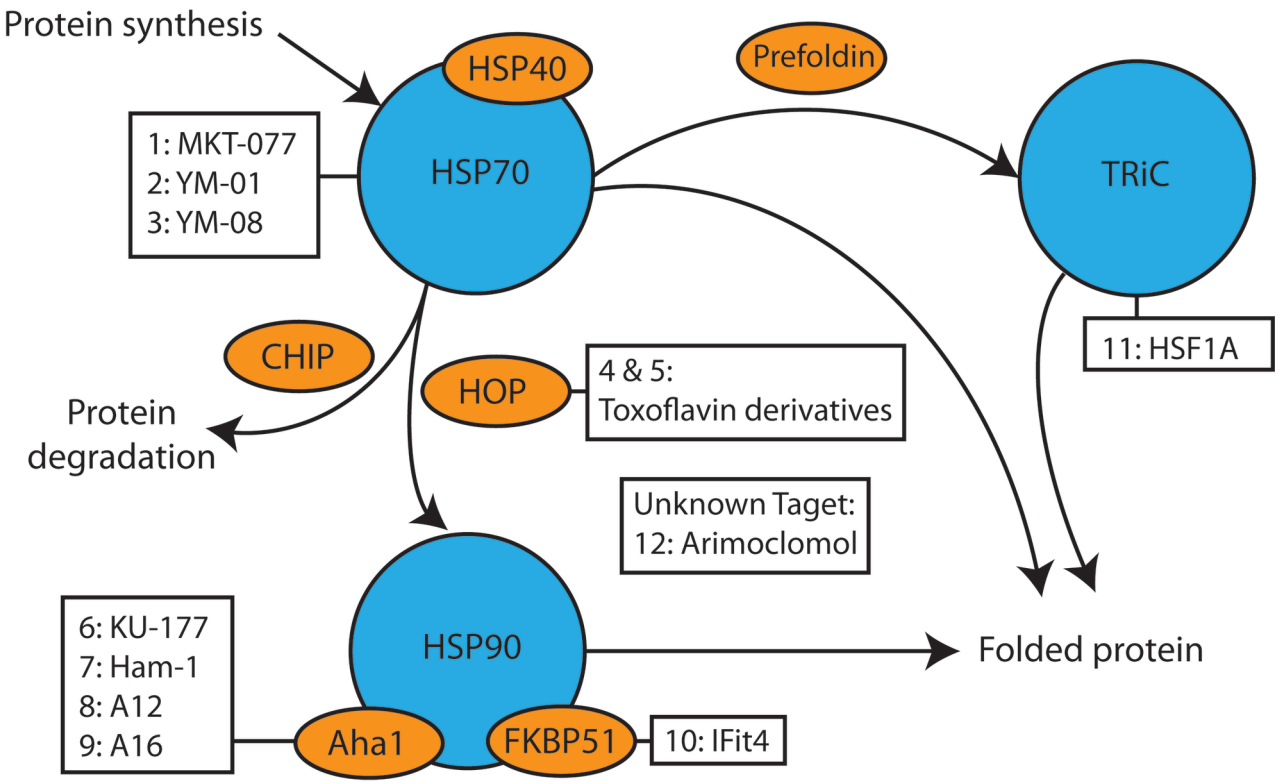

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