JPP JOURNAL OF PROTEINS AND PROTEOMICS
3(2), July-December 2012, pp. 129-139

REVIEW ARTICLE

OSMOLYTE INDUCED STABILIZATION OF PROTEIN MOLECULES: A BRIEF REVIEW

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Abstract: Cells use small molecule osmolytes to protect themselves from harsh environmental conditions. These molecules protect cellular infrastructures against denaturation and aggregation. This is important because aggregation and denaturation of cellular proteins can lead to various physiological malfunctions and life threatening diseases. Osmolytes enhance folding of different proteins, increase their stability and reduce aggregation; and hence are often termed as "chemical chaperones". The present review sheds fresh insights into the properties and mechanism of action of these interesting molecules, along with the recent concept of pharmacological chaperones. The interesting mechanism of a potent protein stabilizer arginine has also been discussed.

Keywords: Osmolytes; chemical chaperones; aggregation; pharmacological chaperones; protein stabilizer.

Protein Misfolding and Conformational Diseases

Native conformation, generally regarded as the most stable conformation at physiological conditions, is responsible for proper functioning of a protein. Misfolded proteins having non-native conformations may not function properly, resulting in different physiological malfunctions (Stefani and Dobson, 2003). Misfolded proteins can also aggregate, resulting in several diseases (Radford and Dobson, 1999). These so called "conformational diseases" can be divided into two broad categories:

(a) Diseases in which a particular protein cannot function properly.

(b) Diseases in which a protein aggregates and form amyloid plaques.

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Received: November 26, 2012
Accepted: November 28, 2012
Published: November 30, 2012

As mentioned above, the first category involves proteins forming incorrect structures (Thomas *et al.*, 1995), or having non-native conformations. There can be many ways by which proteins can form incorrect structures including mutations or chemical modifications (Stefani, 2004). Changes in physiological conditions can also lead to generation of aberrant protein structures. Cystic fibrosis is one of the prime examples of the diseases occurring due to improper protein function.

The second category of the conformational diseases comprises the diseases which occur due to protein aggregation. There are many ways by which a particular protein can aggregate. For example, localized unfolding of native monomers can promote aggregation as seen in the case of many proteins (Sinha *et al.*, 2001; Liu *et al.*, 2001; Sanders *et al.*, 2004; Chow *et al.*, 2004). The reason being, exposure of residues normally protected from solvent or by direct interactions between the unfolded proteins. Changes in net charge, hydrophobicity etc. of a protein can also result in aggregation (Chiti *et al.*, 2003). In addition, protein

aggregation may arise from increased cellular expression of a protein or as a result of folded-partially folded equilibrium being shifted towards partially folded states (Stefani, 2004). Impaired cellular machinery facing overwhelming misfolded or unfolded proteins can drive proteins into the aggregated states.

Conformational transition from α-helices to β-sheets occurs during aggregation of many proteins (Ding *et al.*, 2005). Many proteins self-assemble to form aggregates known as amyloid fibrils (Chong *et al.*, 2011). Amyloids from various proteins have a common core structure composed mainly of β-sheets. The deposition and aggregation of proteins or peptides having the "cross-β" structure is termed amyloidosis. Increased hydrophobic surface area, unsatisfied hydrogen bond donors and acceptors are frequently responsible for increasing amyloidosis of a protein (Liu *et al.*, 2000; Monti *et al.*, 2005; Ahn *et al.*, 2006; Qin *et al.*, 2007; Calabrese *et al.*, 2008). Alzheimer's and Parkinson's diseases and cancers related with aggregation of tumour suppressor protein p53 (Stefani, 2004) are prime examples of diseases occurring due to protein aggregation. On another note, protein aggregation also poses problems during heterologous protein expression in bacteria, and during protein formulation development. Protein aggregation *in vitro* is also responsible for low yields and immunogenic problems associated with protein based formulations.

Stabilization of native state has become a powerful method to suppress protein aggregation (Dobson, 2003). A lot of effort is being directed towards this recently, including the use of protein stabilizers/osmolytes and/or chemical/pharmacological chaperones, and some encouraging progress has been made. We will try to provide a brief outline of the potential these molecules hold in the following sections.

Osmolytes and/or Chemical/pharmacological Chaperones

Cells use two important and effective strategies to survive under stress and to reduce aggregation: (i) the use of efficient large proteins or molecular chaperones, and (ii) accumulation of small molecules known as chemical chaperones or osmolytes.

Molecular chaperones are large protein molecules, which stabilize and/or assist in the correct folding and assembly of unfolded proteins. Many of these are Heat Shock Proteins (HSP's), which are synthesized by cells in response to high temperature or other cellular stresses. They prevent inappropriate association or aggregation of proteins with exposed hydrophobic surfaces.

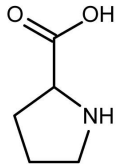
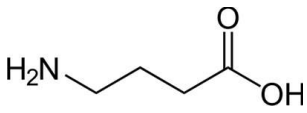
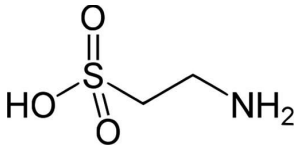
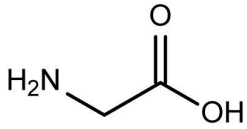
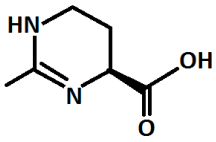
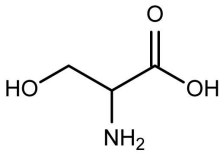
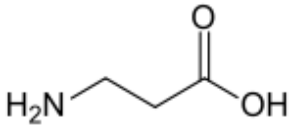
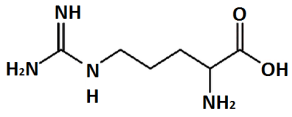
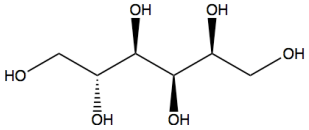
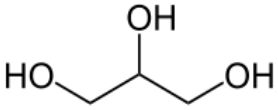
In addition to molecular chaperones, cells also produce or accumulate certain small molecules, known as osmolytes, to combat non-native conditions. Osmolytes enhance protein folding and stability; hence they are often called as "chemical chaperones" or in other words small molecule equivalent of large protein based molecular chaperones (Rajan *et al.*, 2011). Osmolytes protect organisms from high osmotic pressure induced stress (Yancey and Somero, 1979; Yancey *et al.*, 1982), extreme temperatures, perturbing solutes and high hydrostatic pressure (Santos and da Costa, 2002; Yancey, 2001; Adams *et al.*, 2007; Singer and Lindquist, 1998).

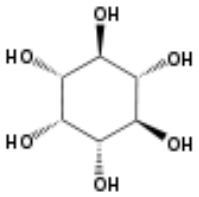
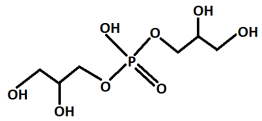
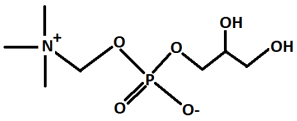
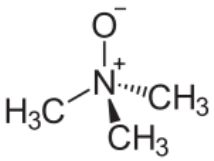
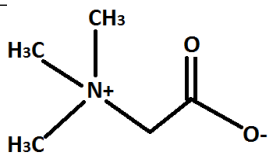
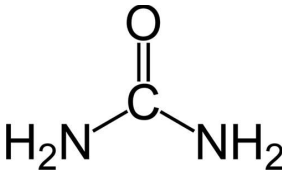
Cellular osmolytes comprise of mainly three classes of organic compounds:

1. Carbohydrates including glycerol, sorbitol and sucrose.
2. Amino acids, such as glycine and alanine.
3. Methylamines, such as betaine and trimethylamine N- oxide (TMAO).

All osmolytes are water soluble and of relatively low molecular mass and include both electrolytes and non-electrolytes. Various osmolyte classes along with some examples are shown in Table 1. Osmolytes are also classified by their protecting and non-protecting natures. Protecting osmolytes do not affect native protein structures (Liang *et al.*, 2010) and have the evolutionary advantage of being compatible at high concentrations with macromolecular structure and function. For example, glycerol, a frequently used protecting osmolyte, does not affect the activity of glucose-6-phosphate dehydrogenase even at concentrations up to 4 M (Borowitzka and Brown, 1974). Non-protecting osmolytes, like urea, on the other hand denatures proteins.

Table 1
Various osmolyte classes along with examples

S.no:	*Osmolyte Class*	*Name*	*Structure*	*Molecular weight (gm/l)*
1.	Amino acids and their Derivatives	Proline		115.13
		Gamma Amino Butyric Acid (GABA)		103.12
		Taurine		125.14
		Glycine		75.06
		Ectoine		142.16
		Serine		105.09
		Beta alanine		89.09
		Arginine*		174.2
2.	Carbohydrates	Sorbitol		182.17
		Glycerol		92.09

		Myoinositol		180.16
		Diglycerol phosphate		246.15
3.	Methylammonium and Methylsulfonium solutes	Glycerophosphorylcholine (GPC)		257.22
		Trimethylamine N-oxide (TMAO)		75.11
		Glycine betaine		117.14
4.	Carbamides	Urea		60.06

*Placed under the category in spite of displaying controversial behavior.

Recently a new class of chemical chaperones has been identified and termed as "Pharmacological chaperones". Osmolytes usually require high concentrations to exert their effects, and sometimes these concentrations can be toxic to cells. Pharmacological chaperones, in contrast, can act at relatively lower concentrations, and are generally safe. Pharmacological chaperones can also target specific proteins and correct their folding defects. This is again in contrast to osmolytes, which non-selectively stabilize proteins (Welch and Brown, 1996).

Mechanism of Osmolyte Action

Bolen and co-workers have proposed the "Osmophobic theory" (Bolen and Baskakov, 2001), which explains the mechanism of osmolyte action on proteins. The 'Osmophobic theory' suggests that a solvophobic thermodynamic force is responsible for the osmolyte action (Bolen and Baskakov, 2001). Strong evidence supporting osmophobic theory has been provided by experimental results (Liu and Bolen, 1995; Qu *et al.*, 1998; Wang and Bolen, 1997). The theory proposes that the property of unfavourable interaction between the osmolyte and the peptide

backbone appears to be selected during evolution for the stabilization of proteins (Bolen and Baskakov, 2001). The basis of this "osmophobic effect" is the finding by Timasheff and colleagues that protecting osmolytes are excluded preferentially from the immediate vicinity of both the native and denatured states of a protein (Arakawa *et al.*, 1990; Lee and Timasheff, 1990). However, the higher exclusion for the denatured states results in much higher increase in its free energy than that of the native state (Fig.1), resulting in the stabilization of the native state (Liu and Bolen, 1995; Qu *et al.*, 1998; Wang and Bolen, 1997; Gekko and Timasheff, 1981; Timasheff, 1993). The degree of preferential exclusion and the increase in the protein chemical potential are directly proportional to the surface area of protein exposed to solvent. Since the unfolded state has more solvent-exposed surface area than the folded state, the free energy gap between the two states is increased. Preferential interaction measurements provide supports that osmolytes are indeed excluded from the protein surface. On the other hand, the favourable interaction and preferential binding of non-protecting osmolyte (for example, urea) with the peptide backbone is thought to be the reason for the denaturing effects of urea on proteins (Wang and Bolen, 1997). To summarize, protecting osmolytes raise the free energy of the unfolded state by unfavourable interactions with the peptide backbone, thus, favouring the folded state. Alternatively, non-protecting osmolytes lower the free energy of the unfolded state, by favourable interactions with the peptide backbone, and favour the unfolded state (Street *et al.*, 2006).

It has been well established that the thermodynamic stabilization of proteins by sucrose is due to preferential exclusion of the sugar from the protein's surface (Lee and Timasheff, 1981). It has been shown clearly that sucrose, a protecting osmolyte, is preferentially excluded from the protein surface, increasing the latter's free energy (Lee and Timasheff, 1981). This leads to thermodynamic stabilization of the protein since the unfolded state becomes less favourable in presence of sucrose (Lee and Timasheff, 1981; Yufeng and Bolen, 1995; Bolen and Baskakov, 2001). Many other reports in the literature confirm the osmophobic effect. For example, reduced and carboxamidated ribonuclease A was shown to expand in urea and contract in protecting osmolytes trimethylamine N-oxide, sarcosine, sucrose, and proline (Qu Y *et al.*, 1998). An early intermediate in the folding pathway of barstar was also shown to be more structured in the presence of osmolytes (Pradeep and Udgaonkar, 2004). Osmolytes have also been found to stabilize the conformations of native proteins (Takano *et al.*, 2004). For example, the stabilization of native proteins against unfolding by sucrose and other molecules has been shown before (Lee and Timasheff, 1981; Arakawa and Timasheff, 1982; Arakawa and Timasheff, 1985). Sucrose was shown to stabilize azurin against unfolding with constant increase in free energy of unfolding with increasing sucrose concentration (Cioni *et al.*, 2005). The increase in free energy of unfolding, for four azurin mutants, was irrespective of differences in their intrinsic stability and internal flexibility indicating that the thermodynamic ability was primarily due to the large surface area of the denatured state (Cioni *et al.*, 2005).

A Molecule of Special Mention: Arginine

Another important protein stabilizer and aggregation suppressor which needs special mention is arginine. There are many controversies surrounding arginine to be considered as a classical osmolyte. Some studies show that it can behave like an osmolyte, whereas others show that it can unfold proteins (Yancey *et al.*, 1982). Arginine has been shown to perturb the stability of enzymes as shown by Yancey *et al.* (Yancey *et al.*, 1982), based on which they concluded that arginine is a protein destabilizer and hence not used by nature as an osmolyte. Similar observation has been made by Xie (Xie *et al.*, 2004). They showed that fluorescence properties of aminoacylase were perturbed by arginine. The protein perturbing nature of arginine is thought to be due to its guanidinium group also present in guanidine hydrochloride (a known protein denaturant). Arginine has also been shown to destabilize cytochrome c during thermal unfolding. Increasing concentration of arginine leads to decrease in both Gibbs energy of stabilization and the midpoint of thermal

transition (Taneja and Ahmad, 1994). Arginine also perturbs aromatic environments in proteins. For example arginine affected the local structures surrounding tyrosine residues in α-crystallin, with little changes in the secondary structure. Hence it may or may not stabilize native proteins, but it does suppress aggregation (Arakawa and Tsumoto, 2003; Ishibashi *et al.*, 2005; Arakawa *et al.*, 2006; Arakawa *et al.*, 2007). Arginine has been shown to facilitate refolding (Ho *et al.*, 2003; Buchner and Rudolph, 1991; Arora and Khanna, 1996; Clark *et al.*, 1999), suppress aggregation (Shiraki *et al.*, 2002; Arakawa and Tsumoto 2003), and to increase reversibility of thermal unfolding (Shiraki *et al.*, 2002; Arakawa and Tsumoto 2003) of a number of proteins. Arginine has also been routinely used to improve refolding efficiency of recombinant proteins expressed in *Escherichia coli,* which would otherwise form inclusion bodies (Buchner and Rudolph 1991; Brinkman *et al.*, 1992; Arora and Khanna 1996; Tsumoto *et al.*, 1998, Umentsu *et al.*, 2003). The arginine solubilized proteins show proper activity and folding, for example green fluorescent protein (Tsumoto *et al.*, 2003b) and β2 microglobulin (Umetsu *et al.*, 2005).

In contrast to osmolytes, the mechanism of action of arginine is somewhat different. It is believed that arginine does not change the equilibrium of the folding process (Shiraki *et al.*, 2002; Taneja and Ahmad, 1994; Arakawa and Tsumoto, 2003). Therefore arginine operates via a different mechanism than osmolytes like sucrose. It only prevents the association of denatured or partially folded protein (Rinas *et al.*, 1990; Buchner and Rudolph 1991; Arora and Khanna, 1996; Armstrong *et al.*, 1999; Baynes *et al.*, 2005). Unlike a protecting osmolyte, arginine may also have significant contributions from direct interactions (Kita *et al.*, 1994). The guanidino group present in arginine is thought to be largely responsible for the strength of these interactions. Arginine has been shown to have a tendency to bind to the protein surface (Rajan *et al.*, 2011). Although guanidinium hydrochloride also interacts with proteins, arginine shows complicated pattern of interactions and is different from guanidinium hydrochloride in its mechanism of action (Arakawa *et al.,* 2007). The binding of arginine to proteins results in the less aggregated state with a greater surface area being favoured. This is thought to increase the solubility of proteins and suppress protein-protein interactions. It is believed that arginine also binds to folding intermediates during refolding and suppresses their aggregation (Tsumoto *et al.*, 2003a). Arginine however has only weak interactions with proteins and is effective at high concentrations (Arakawa *et al.,* 2007). Arginine can interact with the specific functional groups of the polypeptide chain favourably and can destabilize the native protein. However, it can also lower the energetic cost of exposure of extended conformations. This results in reduced aggregation of the protein (Reddy *et al.*, 2005). Arginine is able to strike a balance between, on the one hand, preserving the relative stability of the native state and, on the other hand, stabilizing denatured polypeptides and intermediates in solution in order to prevent them from following the path down to aggregation (Reddy *et al.*, 2005). This property is not shown by the common osmolytes. Baynes *et al.* have proposed "Gap effect theory" (Baynes and Trout, 2004; Baynes, 2005) to describe the mechanism of action of arginine, to counter aggregation. Gap effect theory is based on preferential binding (Timasheff, 1998), osmotic stress (Colombo *et al.*, 1992), and Kirkwood-Buff theory (Kirkwood and Buff, 1951; Shimizu, 2004; Shimizu and Smith, 2004; Smith, 2004) and previous understanding of the effect of additives on protein thermodynamics. They proposed that for arginine to be preferentially excluded from protein complexes, and to reduce aggregation rates without affecting the folding rate and equilibrium, it is necessary that arginine does not interact with single protein molecules differently than water. They have termed arginine as a neutral crowder; it does not shift the energy of isolated protein molecules significantly. Rather, by excluding itself from the inter protein gap in protein-protein complexes, arginine decreases the protein association and dissociation rates. Steric reasons are proposed to be responsible for this effect. Arginine also increases the free energy of the protein-protein encounter complex, making them unstable.

Work has also been undertaken at our laboratory to investigate the mechanism of action of arginine using Fluorescence Correlation

Spectroscopy (FCS) and other biophysical techniques. FCS, being a single molecule technique provides information which may be averaged out in an ensemble measurement. Further, nano-molar sample concentrations needed for a FCS experiment overcomes any aggregation related complication. Using Bovine Serum Albumin as a model system, we have shown that arginine suppresses the aggregation of the protein (Ghosh *et al.*, 2009). Our results show that arginine inhibits formation of partially folded intermediates, potentially involved in the aggregation process. Using FCS we have measured further the rapid microsecond dynamics of a protein which has been found modulated by the presence of arginine (Haldar *et al.*, 2010).

Our results also suggest the possibility of dual behaviour of arginine, functioning both like an osmolyte and a protein denaturant. Arginine behaving like a true osmolyte, contracts the urea unfolded protein, but it also contracts the native protein. A traditional osmolyte, like sucrose, does not offer this behaviour (Haldar *et al.*, 2010). The data show that arginine interacts both with the protein backbone and also with the side chains, while traditional osmolytes do not interact with the side chains (Haldar and Chattopadhyay, 2011).

Osmolytes and/or Chemical/pharmacological Chaperones as Inhibitors of Protein Misfolding and Aggregation

Osmolytes have been used frequently to correct misfolding and to counter aggregation of various proteins *in vitro*. They are also commonly used to stabilize protein-based biopharmaceuticals such as recombinant human interferon and various therapeutic antibodies (Besman *et al.*, 2008; Carpenter *et al.*, 2008; Webb *et al.*, 2003; Chi *et al.*, 2005), and protein-based vaccines such as those for hepatitis A and B (Brandau *et al.*, 2003). Arginine has been shown to suppress the aggregation of many partially unfolded proteins during thermal unfolding and refolding (Arakawa and Tsumoto, 2003; Shiraki *et al.*, 2002; Kudou *et al.*, 2003; Reddy *et al.*, 2005; Tsumoto *et al.*, 2004). More specifically, arginine was shown to suppress aggregation of interleukine-6 (IL-6) and a monoclonal antibody (mAb) during thermal unfolding, in a concentration dependent manner (Arakawa *et al.*, 2006). It also suppressed the aggregation of denatured hen egg white lysozyme (Reddy *et al.*, 2005) and was found to be the most effective amino acid in suppressing the aggregation of heat-denatured protein (Shiraki *et al.*, 2002).

Unfortunately, despite their many in vitro applications, the in vivo use of osmolytes or other protein stabilizers has been difficult mainly because of the high concentrations needed (Arakawa *et al.*, 2006). Nevertheless, recent work has shown that these molecules can be used *in vivo* also. For example it has been shown that glycerol and trimethylamine N-oxide (TMAO) can correct the temperature-sensitive folding defect of the human cystic fibrosis transmembrane conductance regulator (CFTR) mutant protein, ΔF508 CFTR (having a deletion of a phenylalanine residue at position 508). Also, Glycerol and TMAO have also been shown to correct the folding defect of the tumour suppressor protein, p53, mutant A125V, in cells. This mutation results in a temperature-sensitive folding defect, which is alleviated by the osmolytes and their effects are reversible (Michalovitz *et al.*, 1990; Martinez *et al.*, 1991). It has been shown that the small molecule 1-deoxy-alactonojirimycin (DTB), acting like a pharmacological chaperone, rescues the mutant proteins responsible for Fabry-disease. These mutations cause accumulation of un-degraded glycosphingolipids and result in stroke or myocardial infarction. The intracellular activity of misfolded variants like N370S in lysosomal storage disease named as Gaucher's disease can be partly restored by administering cell-permeable b-Glu inhibitors that serve as chemical/pharmacological chaperones. Aggregation and cytotoxicity induced by expanded polyglutamine stretch in protein ataxin-3 involved in Machado-Joseph disease, has been shown to be decreased by Glycerol and TMAO (Yoshida *et al.*, 2002). Trehalose has been shown to prevent fibrillation and cytotoxity of AB-peptide and to protect prion-infected cells from induced oxidative damage (Liu *et al.*, 2005; Berangar *et al.*, 2008). TMAO has been shown to prevent misfolding of prion proteins (Bennion *et al.*, 2004). Trehalose has been shown to bind

partially folded polyglutamine proteins and stabilize them, in turn alleviating the symptoms of Huntington's disease in a mouse model (Davies *et al.*, 2006). Osmolytes have been used frequently in preserving living biological materials, for example organ storage for transplants (Wiggins *et al.*, 1999).

Although the use of osmolytes has been common as aggregation inhibitors, contrasting results are also available. For example, Trehalose was shown to delay thermal unfolding of cutinase, but on the other hand favoured pathways leading to irreversible denaturation of the protein (Baptista *et al.*, 2008). Fibrillation of carboxymethylated bovine α-lactalbumin was shown to be enhanced by TMAO and sucrose (Bomhoff *et al.*, 2006). Microtubule associated protein tau fibrillation was found promoted by TMAO (Scaramozzino *et al.*, 2006).

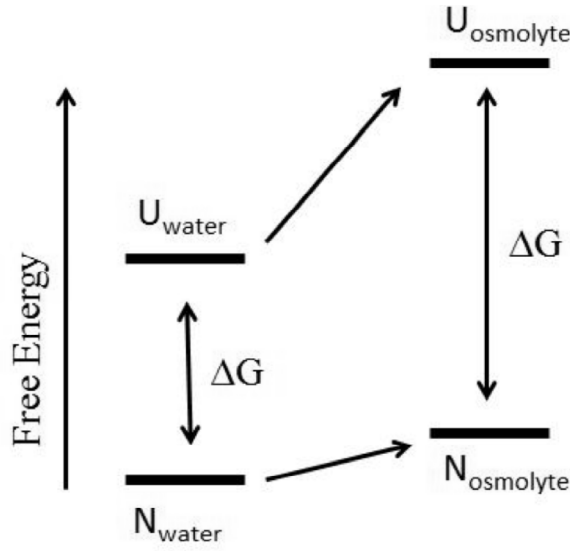


Figure 1: **Difference in free energies of native and unfolded states in the presence and absence of a protecting osmolyte. N and U denote native and unfolded states respectively. Protecting osmolytes interact unfavorably with both N and U states. However the strength of the interaction is much more for U (due to its large surface area) resulting in its destabilization.**

Conclusion

The potential of protecting osmolytes and/or chemical chaperons as protein stabilizers has been further extended by the ever increasing popularity of protein related biologics pharmaceuticals. These molecules are inherently complex with challenging stability profiles. Designing and developing formulations, particularly in the liquid state, would need extensive understanding of the small molecule stabilizers using chemical, physical and biological tools. The advent and popularity of 'chemical biology' methodologies is expected to expedite that process.

Abbreviations

p53, tumour protein 53; HSP, Heat Shock Protein; TMAO, Trimethylamine N-oxide; FCS, Fluorescence Correlation Spectroscopy; IL-6, Interleukin-6; mAb, Monoclonal antibody; CFTR, Cystic Fibrosis Transmembrane Conductance Regulator; DTB, 1-deoxy-alactonojirimycin; b-Glu, β-Glucosidase enzyme; AB-peptide, Amyloid-beta peptide.

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