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REVIEW

The endoplasmic reticulum protein folding factory and its chaperones: new targets for drug discovery?

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Cytosolic heat shock proteins have received significant attention as emerging therapeutic targets. Much of this excitement has been triggered by the discovery that HSP90 plays a central role in the maintenance and stability of multifarious oncogenic membrane receptors and their resultant tyrosine kinase activity. Numerous studies have dealt with the effects of small molecules on chaperone- and stress-related pathways of the endoplasmic reticulum (ER). However, unlike cytosolic chaperones, relatively little emphasis has been placed upon translational avenues towards targeting of the ER for inhibition of folding/secretion of disease-promoting proteins. Here, we summarise existing small molecule inhibitors and potential future targets of ER chaperone-mediated inhibition. Client proteins of translational relevance in disease treatment are outlined, alongside putative future disease treatment modalities based on ER-centric targeted therapies. Particular attention is paid to cancer and autoimmune disorders via the effects of the GRP94 inhibitor geldanamycin and its population of client proteins, overloading of the unfolded protein response, and inhibition of members of the IL-12 family of cytokines by celecoxib and non-coxib analogues.

Abbreviations

BAP, BiP-associated protein; CNX, calnexin; CRT, calreticulin; CST, castanospermine; dNJ, deoxynojirimycin; ERAD, endoplasmic reticulum associated degradation; ERdj, endoplasmic reticulum DNAJ-like; ERQC, endoplasmic reticulum quality control; GRP, glucose regulated protein; HSP, heat shock protein; IGF, insulin growth factor; IL, interleukin; PDI, protein disulphide isomerase; TFM-C, trifluoromethyl-celecoxib; TLR, toll-like receptor; UDP, uridine diphosphate; UPR, unfolded protein response

Introduction to the ER

The ER is home to an array of interlinked chaperone proteins upon which correct folding, partner chain assimilation and final multimer assembly of secreted proteins depend. This can be broken down into a number of semi-distinct functional systems. The lectin-binding chaperone system, consisting of calreticulin (CRT) and the membrane-bound homologue calnexin (CNX) operate in tandem with the *N*-glycan processing enzymes glucosidase I, glucosidase II and quality control checkpoint uridine diphosphate (UDP)-glucose glycoprotein glucosyltransferase (UGGT), to facilitate glycoprotein folding (Moremen and Molinari, 2006). The ER is also home to a multichaperone 'glucose regulated protein (GRP)' complex homologous to the cytoplasmic heat shock protein (HSP) complex of HSP90/HSP70. This ER complex centres on the HSP70 homologue GRP78 (Hendershot, 2004) and the HSP90 homologue GRP94 (Argon and Simen, 1999), but has been found to associate with a collection of ER DNAJ like (ERdj) HSP40 like co-chaperones (Shen and Hendershot, 2005; Dong *et al.*, 2008) and peptidylpropylisomerases (Meunier *et al.*, 2002) of similar ilk to those present in the HSP90/HSP70 complex, as well as with the two GRP78 nucleotide exchange factors BiP-associated protein (BAP) (Chung *et al.*, 2002) and GRP170 (Weitzmann *et al.*, 2006).

Operating both in tandem and independently of the lectin and GRP systems are the protein disulphide isomerase (PDI) family of disulphide bond oxidase, reductase and

isomerase enzymes. ERp57 is found in direct association with CRT and CNX in catalysis of glycoprotein disulphide bond processing. PDIA2, ERp72 and PDIA6 have all been found to operate under the auspices of the GRP multichaperone complex, though functionally have also been observed to operate independently in both chaperone and ER regulatory functions (Meunier *et al.*, 2002; Maattanen *et al.*, 2006; Appenzeller-Herzog and Ellgaard, 2008).

This array of chaperone systems, with varying overlapping functions and interdependencies, has until now been mainly investigated in the interests of the basic mechanistics of ER folding and quality control. This work has more recently begun to give way to the discovery of a number of novel ER targeted compounds which are capable of drug-induced retention of specific pools of client proteins. This opens the way to the exploration of the ER as a therapeutic avenue for disease amelioration via specific drug-induced retention of etiologically significant proteins based on their ER chaperone dependence.

Drugging the lectin binding glycoprotein system

The mechanistics of lectin-binding proteins and sugar-processing enzymes in folding and ER quality control (ERQC) have been comprehensively summarised elsewhere (Anelli and Sitia, 2008), an overview of which is illustrated in Figure 1. Various archetypal inhibitors of these processes have been discovered and utilised for the elucidation of glycoprotein progression in the early folding stages of the secretory pathway. These are: thapsigargin, which reduces ER Ca^{2+} levels via inhibition of Ca^{2+} ATPases (Thastrup *et al.*, 1990); tunicamycin, an inhibitor of *N*-glycan preassembly (Kuo and Lampen, 1974); the glucosidase I and II inhibitors castanospermine (CST) and 1-deoxynojirimycin (dNM) which block deglucosylation of *N*-glycan side chains (Oliver *et al.*, 1997), the site of action of these compounds is shown in Figure 1.

The complex, secreted glycoproteins thyroglobulin (Di Jeso *et al.*, 2005), preprolactin (Oliver *et al.*, 1997) and the homodimer interferon-γ (IFN-γ) (Vandenbroeck *et al.*, 2006) have been used to study folding in the ER in the presence of these inhibitors. CST results in a significant decrease in CRT and ERp57 interaction with thyroglobulin and preprolactin. ER Ca^{2+} depletion by thapsigargin induces early release of thyroglobulin from CRT and CNX, increased retention time on GRP94 and GRP78 and failure of thyroglobulin export to the Golgi (Di Jeso *et al.*, 1999; 2003). While the goals of these studies were purely mechanistic, they indicated the potential for ER targeting to induce altered ER chaperone association and induce cellular retention of complex proteins.

Less complex unglycosylated proteins such as albumin are unaffected by perturbing ER calcium homeostasis (Alloza *et al.*, 2006) and CRT/CNX function (Wong *et al.*, 1993). Conversely, secretion of highly glycosylated heterodimeric antibodies such as IgG_1 is unaltered by ER calcium perturbation (McLaughlin *et al.*, 2010), or tunicamycin (Hashim and Cushley, 1988), while IgM is unaffected by ER or Golgi glycosidase inhibitors (Hashim and Cushley, 1988) but appears susceptible to calcium perturbation (Shachar *et al.*, 1992). This early work, in conjunction with cellular knockout studies (Molinari *et al.*, 2004), illustrated that large-scale disruption of ER chaperones was not as deleterious to cell growth and survival as would first be anticipated.

Glucosidase inhibitors as antivirals

Many viral particles consist of an RNA or DNA genome, enclosed within a protein capsid and outer glycoprotein-containing envelope. This envelope serves the function of host cell recognition, membrane fusion and entry of the viral genome to the cell. The majority of antiviral therapies target intrinsic viral targets, such as neuraminidase or reverse transcriptase. An alternate approach is to target extrinsic mechanisms of the host essential to the viral life cycle, such as folding/assembly in the secretory pathway. Bovine viral diarrhoea virus (BVDV) when entrapped in the ER by brefeldin A, still assembles into fully infectious viral particles (Macovei *et al.*, 2006). This data may pinpoint the ER rather than the Golgi as the cellular compartment of consequence for the development of viral infectivity.

Compounds under the umbrella of the α-glucosidase inhibitors such as castanospermine (CST), 1-deoxynojirimycim (dNJ) and their analogues 6-O-butanoylcastanospermine (BuCast), *N*-butyl (*N*B-dNJ) and *N*-nonyldeoxynijirimycin (*N*N-dNJ) have been shown to alter export or cause detrimental reduction in viral infectivity of the following: hepatitis B (HBV) (Lazar *et al.*, 2007), hepatitis C (HCV) (Chapel *et al.*, 2007), bovine viral diarrhoea virus (BVDV) (Durantel *et al.*, 2004), dengue fever virus (DEN1-4) (Schul *et al.*, 2007), herpes simplex virus (Bridges *et al.*, 1995), HIV-1 and HIV-2 (Pollock *et al.*, 2008), influenza (Pieren *et al.*, 2005), parainfluenza virus type 3 (Tanaka *et al.*, 2006), Japanese encephalitis virus (Wu *et al.*, 2002), measles (Bolt *et al.*, 1999), Rauscher murine leukaemia (Ruprecht *et al.*, 1989), rubella (Nakhasi *et al.*, 2001) and Sindbis virus (Schlesinger *et al.*, 1985).

The antiviral effect of α-glucosidase inhibitors has been confirmed *in vivo* against all dengue virus serotypes (Whitby *et al.*, 2005; Schul *et al.*, 2007), herpes simplex virus strain SC16 (Bridges *et al.*, 1995), Japanese encephalitis virus (Wu *et al.*, 2002), Rauscher murine leukemia virus (Ruprecht *et al.*, 1989) and woodchuck hepatitis virus (Block *et al.*, 1998). CST is, however, not universally broad spectrum, having minimal antiviral impact against yellow fever virus, West Nile virus (Whitby *et al.*, 2005) and some but not all strains of vesicular stomatis virus (VSV) (Schlesinger *et al.*, 1984). It can be postulated that this is due to these strains containing viral glycoproteins that are capable of competent folding without the need for CRT/CNX; however, concrete investigations have not been carried out to verify if this is the case.

Comparative studies against existing antiviral treatments show that *in vivo* viremia of dengue fever virus serotype 2 in mice is reduced by 93% and 88%, respectively, with *N*N-dNJ and BuCast. This effect was greater than the viral RNA replication inhibitors 7-deazamethyladenosine (70%) and ribavirin (no effect) (Schul *et al.*, 2007). Similar results were also obtained for BVDV, a surrogate *in vitro* model of HCV infection, with IFN-γ and ribavirin (Ouzounov *et al.*, 2002; Durantel *et al.*, 2004). While *in vitro* removal of IFN-γ and ribavirin results in immediate rebound of BVDV viral production (Woodhouse *et al.*, 2008), addition of *N*B-dNJ at physiologically

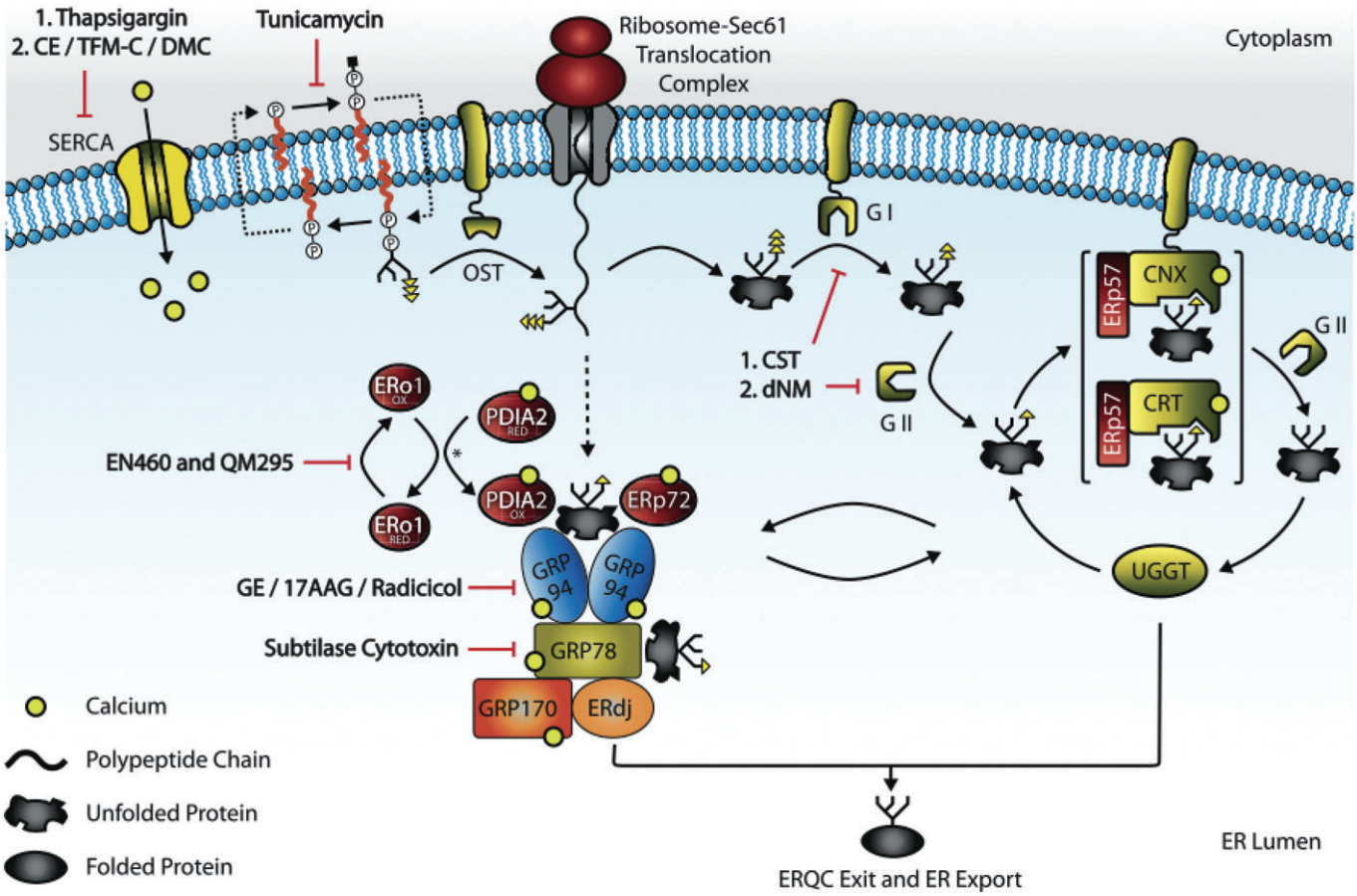

Figure 1

Schematic representation of ER folding pathways and established sites for pharmacological inhibition. Protein translation results in polypeptide entry into the ER where oligosaccharyltransferase (OST) recognises and transfers preassembled $Glc_3Man_9GlcNAc_2$ structures from dolichol, a polyisoprenoid lipid membrane anchor molecule, onto Asn-X-Ser/Thr *N*-glycosylation sites. Two of three terminal glucose residues are sequentially cleaved by glucosidase I (GI) and glucosidase II (GII) allowing glycoprotein interaction with the lectin binding calreticulin (CRT) and calnexin (CNX) system. Dotted arrow shows that GRP78 may also interact with polypeptide chains upon initial entry into the ER. Exit from CRT/CNX is followed by cleavage of the final third glucose by GII. UDP-glucose glycoprotein glucosyltransferase (UGGT) acts as a folding sensor and is able to reglucosylate unfolded proteins for re-entry to the CRT/CNX folding cycle. Cargo proteins may also interact with the multimeric glucose-regulated protein (GRP) chaperone group comprising GRP78, GRP94 and the co-chaperones GRP170, BAP (not shown) and ERdj1-7. GRP78 and GRP94 possess weak ATPase activity and are capable of binding unfolded client proteins, preventing aggregation and promoting correct folding. ERdj HSP40-like co-chaperones promote ATP hydrolysis of GRP78 with GRP170 and BAP acting as GRP78 nucleotide exchange factors. Currently, no GRP94 co-chaperones have been identified. PDI family members PDIA2 and ERp72 are capable of operating independently or concurrently with GRP-protein complexes. Cycling of the oxidoreductase Ero1 enables oxidation of $PDIA2_{red}$ to $PDIA2_{ox}$ facilitating disulphide bond formation. While PDIA2 has been illustrated (*), the ability of Ero1 to maintain a functional redox state is shared with other ER protein disulphide isomerases. Correctly folded proteins which are no longer captured by components of the CRT/CNX system or GRP complex are dubbed to have 'passed' ER quality control, allowing exit from the ER to the Golgi.

tolerated concentrations was capable of complete BVDV (Woodhouse *et al.*, 2008) and HCV (Steinmann *et al.*, 2007) viral clearance. Reduction in viremia by *N*N-dNJ and viral clearance has also been shown *in vivo* in woodchucks chronically infected with woodchuck hepatitis virus (Block *et al.*, 1998).

Glucosidase inhibitors compromise viral infectivity

Mechanistically, the antiviral effect of α-glucosidase inhibitors is composed of two ER-centric mechanisms. First, both BVDV (Jordan *et al.*, 2002) and HBV (Block *et al.*, 1994) exhibit decreased viral release which does not correspond to any decrease in viral genome replication. Only dengue fever virus serotype 2-infected cells show reduction in viral RNA synthesis in response to α-glucosidase inhibitors (Wu *et al.*, 2002). Degradation, putatively via the ER-associated degradation (ERAD)-proteasome pathway, is indicated through studies of HBV with ER mannosidase inhibitors (Branza-Nichita *et al.*, 2002) or proteasomal inhibitors (Simsek *et al.*, 2005). Inhibition of either results in attenuation of α-glucosidase inhibitor-induced degradation of HBV glycoproteins. The second more intriguing ER mechanism was that

of the viral particles still released, their infectivity was highly compromised. Of the viruses listed previously susceptible to α-glucosidase inhibitors, viral particles still capable of release under conditions of drug treatment were universally found to have reduced infectivity. This is true for HBV (Lazar *et al.*, 2007), HCV (Chapel *et al.*, 2007), dengue virus serotype 2 (Whitby *et al.*, 2005), BVDV (Durantel *et al.*, 2001), HIV (Papandréou *et al.*, 2002) and parainfluenza type 3 (Tanaka *et al.*, 2006).

The mechanism by which this occurs has been elucidated in measles, HIV and HCV virus-like particles (HCV_{VLP}). HCV_{VLP} when treated with alkyl chain dNJ derivates exhibit impaired binding properties to target hepatocytes (Chapel *et al.*, 2006). The drug-induced conformation of the HCV_{VLP} glycoprotein E2 differed from the natural one when probed by either linear sequence or conformationally sensitive epitope targeting antibodies, corresponding to loss of infectivity (Chapel *et al.*, 2007). Loss of conformation-dependent antibody recognition was also observed for F protein in measles and gp120/gp41 env in HIV-1, with trafficking to the cell surface unaltered. Both F protein and gp120/gp41 were still capable of binding CD46 and CD4 target proteins, respectively, but neither was capable of initiating membrane fusion (Bolt *et al.*, 1999; Papandréou *et al.*, 2002).

Independent studies show that upon CNX knockout, CRT interacts with HA, not in the capacity of 'folding facilitator' but in that of 'controller of quality of folding' where it acts to retain HA in the ER, unable to fold competently in the absence of CNX. Use of CST blocks both CNX and CRT interaction preventing this quality control role, i.e. without interception by a chaperone in the ER, HA is allowed to be secreted, but this secreted form is improperly folded (Molinari *et al.*, 2004; Pieren *et al.*, 2005). Therefore, viral particles are compromised in infective potency due to atypical envelope glycoprotein conformation facilitated through escape from ERQC, illustrated in Figure 2.

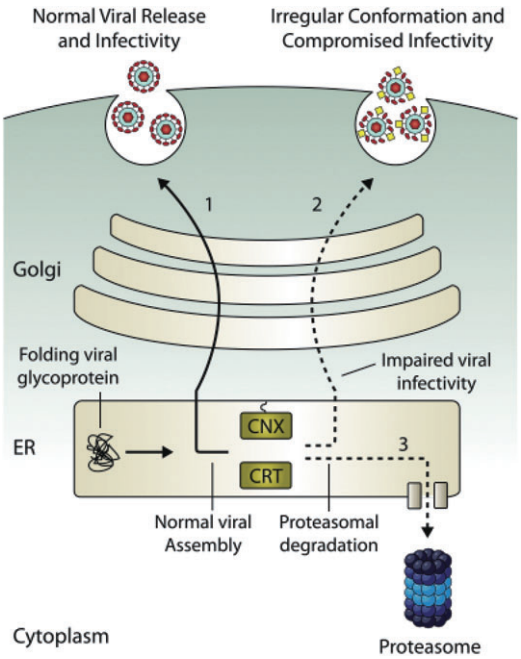

Figure 2

Glucosidase inhibitors can inhibit viral export and infectivity. During the normal viral trafficking and budding pathway, viral glycoproteins fold and assemble in the ER dependent on the chaperones CRT and CNX, and viral particles bud from the Golgi before release from the cell (1). Viral folding and assembly under α-glucosidase exposure (dotted arrows) results in two viral glycoprotein populations, both incapable of interacting with CRT/CNX. While one subset is incorrectly folded to an extent that ERQC chaperones apart from CRT/CNX may successfully intercept and target it for proteasomal degradation (3), the other manages to gain a sufficiently competent state to allow it to evade ERQC (2), and assembles into viral particles for export. This latter population, while capable of evading ERQC, appears sufficiently different from that of the CRT/CNX-dependent native conformation to be unable to initiate host cell fusion and entry.

Glucosidase inhibitors in clinical trials

High toxicity or significant side effects are not a barrier to clinical use as *N*B-dNJ (Miglustat) is currently on the market for the treatment of Gaucher's disease acting as a stabilising 'chemical chaperone' for mutant β-glucosidase (Sawkar *et al.*, 2002). In human anti-HIV-1 trials of *N*B-dNJ, the most common side effects were diarrhoea and flatulence (Tierney *et al.*, 1995), identical side effects to those observed in all Gaucher's disease clinical trials (Cox *et al.*, 2000; Heitner *et al.*, 2002) with symptoms ending upon termination of treatment.

In terms of efficacy in human clinical trials against HIV-1 infection, one study observed no trend in HIV-1 marker p24 or CD4 + T cell count, though the maximum tolerated dose was not used as the study was discontinued early (Tierney *et al.*, 1995). Increased CD4 counts and suppression of HIV-1 p24 were observed in another trial using a higher dosage (Fischl *et al.*, 1994). Methods to overcome problems with high dosage have been studied *in vitro* using targeted delivery. CD4 linked liposomes loaded with *N*B-dNJ targeted to the gp120/gp41 complex of HIV-1 and HIV-2 induced 100 000-fold reduction in IC_{50} values from the high $\mu mol \cdot L^{-1}$ of free *N*B-dNJ to low $nmol \cdot L^{-1}$ range for targeted encapsulation (Pollock *et al.*, 2008).

Antivirals targeting the ER may also attenuate problems related to resistance in existing antiviral strategies such as the seasonal flu vaccine (Russell *et al.*, 2008). Mutation of exposed viral amino acids may lead to evasion of any previously generated immune recognition. As viral glycoprotein folding has been shown to be dependent on the retention of asparagine residues at sites of *N*-glycosylation (Hebert *et al.*, 1997), CRT/CNX-independent drug-resistant variants are less likely to emerge as a consequence of viral mutation.

Proteostasis, mutant aggregates and targeted folding

The term proteostasis refers to the biological machinery integrating protein synthesis, folding, quality control, trafficking and degradation (Anelli and Sitia, 2010). Rebalancing potential deficiencies in proteostasis characteristic of metabolic,

neurodegenerative, cardiovascular disorders or cancer is thought to be achievable through pharmacological or biological manipulation. The above mentioned use of *N*B-dNJ as a chemical chaperone of mutant β-glucosidase indicates that targeting of the ER can also encompass pharmacological agents designed to promote specific folding of otherwise folding-incompetent proteins. The serpinopathies constitute a disease type characterised by mutation of serpins such as α1-antitrypsin (α1AT) resulting in both toxic ER retention/aggregation and loss of serine protease inhibition (Ekeowa *et al.*, 2009). This loss of function, gain of aggregate toxicity is also a feature of hereditary hemochromatosis (De Almeida and De Sousa, 2008) and primary open angle glaucoma (Stone *et al.*, 1997). Use of chemical chaperones such as 4-phenylbutyric acid can rescue mutant α1AT to 20–50% of wild type levels *in vivo* (Burrows *et al.*, 2000). A comprehensive summary of proteostatic manipulation is available elsewhere (Balch *et al.*, 2008).

Indirect chaperone disruption by celecoxib

As a site of cellular calcium storage, the majority of ER chaperones bind calcium or actively require calcium in order to bind and release client proteins (Nigam *et al.*, 1994; Biswas *et al.*, 2007) (see Figure 1) presenting ER calcium perturbation as an alternate avenue for the indirect modulation of chaperone function. Pfizer's Celebrex® (celecoxib), a nonsteroidal anti-inflammatory drug, was originally designed to specifically inhibit the COX-2 isoform of prostaglandin H synthase upregulated at sites of inflammation and cancer, but a growing list of findings shows many COX-2 independent functions attributable to celecoxib (Johnson *et al.*, 2002; Alloza *et al.*, 2006; Lou *et al.*, 2006). It is now becoming clear that many of these endpoints are mediated through alteration of calcium homeostasis of the ER, and as such, this group of drugs may be considered the standard bearer for translational exploitation of the ER chaperone environment in applications related to both autoimmunity and cancer.

The ability of celecoxib to increase cytoplasm calcium concentrations has been demonstrated in numerous cancer and noncancer cell lines (Alloza *et al.*, 2006; Tsutsumi *et al.*, 2006; Pyrko *et al.*, 2007). Experiments with other COX-2 inhibitors have demonstrated that the ability to alter calcium levels is unique to celecoxib and not a shared generic characteristic of COX-2 inhibitors (Johnson *et al.*, 2002; Alloza *et al.*, 2006). Celecoxib has been shown to inhibit ER Ca^{2+} ATPase pumps and when compared with the known Ca^{2+} ATPase inhibitor thapsigargin, has a similar Ca^{2+} ATPase activity profile, but much less potent IC_{50} value of 35 μM compared with 29 nM for thapsigargin (Johnson *et al.*, 2002).

Proteomic analysis of the COX-2 deficient HCT-116 colorectal cancer cell line has revealed that celecoxib elicits numerous fluctuations in intracellular protein levels, including the ER chaperones GRP78 and GRP94 (Lou *et al.*, 2006); however, this study did not scrutinize changes linked to the secretome. Alongside investigations in COX-2 deficient cells, a new generation of 'non-coxib' celecoxib analogues devoid of COX-2 inhibitory action has been developed. Most prevalent in the literature is 2,5-dimethyl-celecoxib (DMC) and 4-trifluoromethyl-celecoxib (TFM-C), both are substantially less potent against COX-2 yet retain the ability to perturb ER calcium (Alloza *et al.*, 2006; Pyrko *et al.*, 2007). Unlike celecoxib, TFM-C has been studied not only on intracellular proteins but also on secreted proteins, with work in our lab revealing its potential in inducing intracellular retention of the heterodimeric IL-12 family of cytokines.

Celecoxib and IL-12 family cytokines

IL-12 family cytokines link the innate systems of macrophage and dendritic cells to that of the adaptive T cell response. Occurring at a critical juncture in the immune response, their dysregulation has been implicated in the etiology of autoimmune disorders such as multiple sclerosis, rheumatoid arthritis and Chrohn's disease. Celecoxib and TFM-C have been shown to inhibit the secretion of the dimeric cytokines IL-12, IL-23 and IL-12p80 while having a negligible impact on monomeric IL-12p40 secretion (Alloza *et al.*, 2006; McLaughlin *et al.*, 2010). This susceptibility was originally postulated due to the unique homo/heterodimeric structure of the IL-12 family, an uncommon characteristic amongst cytokines. This follows the principle of what is suspected to be the gamut of secretory proteins susceptible to ER retention, i.e. the greater the complexity of folding, the greater the dependence on chaperone function for successful ER export. The faster kinetics for the appearance of secreted recombinant IL-12p40 following transcriptional induction compared with IL-12p80 gives further weight to the concept of elevated folding complexity linked to drug susceptibility (Alloza *et al.*, 2006).

At the minimal concentrations still capable of inhibiting IL-12 secretion, neither celecoxib nor TFM-C seems to affect cell viability or transcription of p40 and p35 chains (Alloza *et al.*, 2006; McLaughlin *et al.*, 2010). The potent COX-2 inhibitor rofecoxib does not affect calcium homeostasis and has no effect on IL-12 family secretion, while thapsigargin is capable of mimicking celecoxib- and TFM-C-induced IL-12 family retention. The evidence converges upon a mechanism by which celecoxib and TFM-C disrupt successful protein folding and dimer assembly based on the complexity of ER posttranslational modification, assembly and dimerisation, via calcium perturbation and not via COX-2 inhibition, nor any other functions attributed to celecoxib (Johnson *et al.*, 2002; Alloza *et al.*, 2006). The currently elucidated but as yet rudimentary ER 'foldosome' of the IL-12p40 subunit, and its altered interactions upon TFM-C drug-induced retention and degradation, are shown in Figure 3.

Celecoxib, the unfolded protein response (UPR) and ERAD

The ER chaperones/factors GRP78, GRP94, GRP170, ERp72, ERdj4, CRT and HERP are all upregulated by 50 μM TFM-C in HEK293 cells (McLaughlin *et al.*, 2010), and all are identified to be under the control of the UPR-activated ER stress response (Yoshida *et al.*, 1998; Lee *et al.*, 2003; Nozaki *et al.*, 2004; Liang *et al.*, 2006). 50 μM TFM-C fails to trigger UPR-induced inhibition of general protein synthesis in HEK293

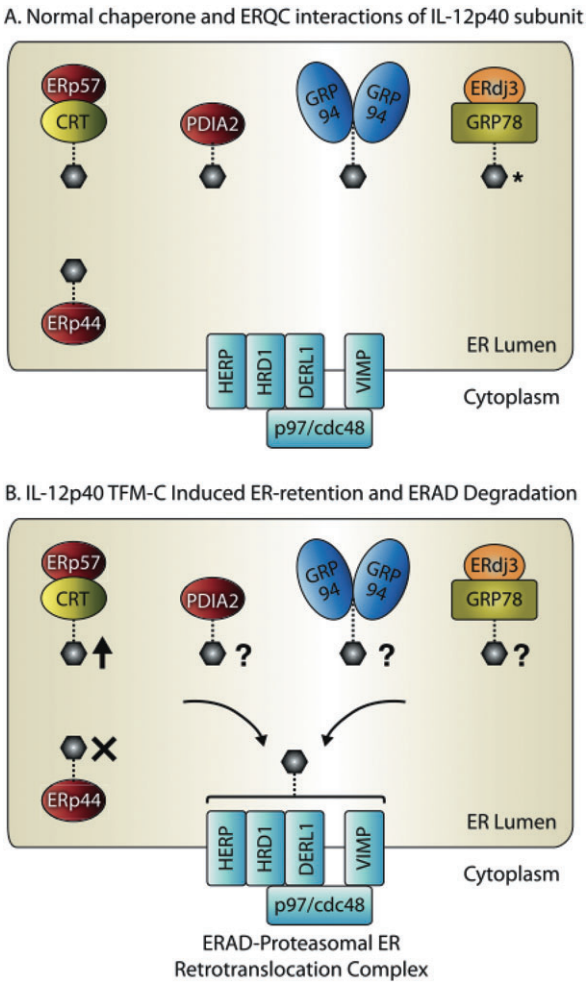

Figure 3

TFM-C-induced alteration to IL-12p40 chaperone interactions. (A) Under normal circumstances the IL-12 family subunit p40 has been shown to interact with CRT, PDI, GR94, GRP78 [*inferred from ability of bacterial HSP70 DnaK to assemble IL-12p40 into IL-12p80 (Martens *et al.*, 2000)] and ERp44. (B) TFM-C results in increased IL-12p40 ERQC capture by CRT and blocks association with ERp44. Modulation of PDI, GRP94 and GRP78 levels are as yet unknown. TFM-C induces IL-12p40 degradation via drug-dependent association with HERP and putatively the rest of the ERAD retrotranslocation proteasomal pathway (McLaughlin *et al.*, 2010).

cells, confirmed by the persistence of retained p19 and p80 under 50 μM TFM-C treatment and by induction of HERP protein (see below) (Alloza *et al.*, 2006; McLaughlin *et al.*, 2010). Evidence of upregulation of many of these chaperones already exists for celecoxib and DMC, these include; GRP94 (Lou *et al.*, 2006; Namba *et al.*, 2007), GRP78 (Namba *et al.*, 2007; Pyrko *et al.*, 2008), GRP170, CRT, CNX (Namba *et al.*, 2007), ERdj3, ERdj4 (Tsutsumi *et al.*, 2006) and PDIA3 precursor (Heum Park *et al.*, 2006).

An intriguing finding was the strong induction of the ER membrane protein HERP by TFM-C (McLaughlin *et al.*, 2010). HERP is ubiquitously expressed and upregulated via an ER stress response element as part of the UPR (Kokame *et al.*, 2000). HERP has been linked to the endoplasmic reticulum associated degradation (ERAD) pathway as part of a large ER membrane retrotranslocation complex, which extracts misfolded ER proteins to the cytosol for proteasomal degradation. This complex consists of HRD1, Derlin-1 like protein (DERL-1), VIMP and p97 (Ye *et al.*, 2004; Schulze *et al.*, 2005) and is linked to cytosolic ubiquilins which shuttle ubiquitinated proteins to the proteasome (Kim *et al.*, 2008).

Notwithstanding that celecoxib and TFM-C induce ER retention of IL-12 cytokines, intracellular p40 and p19 remain relatively constant (Alloza *et al.*, 2006; McLaughlin *et al.*, 2010). This lack of accumulation against the background of ongoing protein synthesis would indicate that IL-12 family subunits undergo degradation and clearance by a cellular degradation pathway. Concurrent with its substantial upregulation by TFM-C, immunoprecipitation shows p40 subunit interaction with HERP, but only in the presence of TFM-C. This is unique in that IL-12p40, unlike other HERP-interacting ERAD substrates such as CD3-delta (Schulze *et al.*, 2005; Kim *et al.*, 2008), connexin 43 (Hori *et al.*, 2004) and the nonglycosylated BiP substrates Ig κ LC, γ LC and λ LC (Okuda-Shimizu and Hendershot, 2007), is the first protein to be shown to interact with HERP only upon drug-induced ER perturbation. HERP knockout increases susceptibility to ER stress and also impedes the degradation of IL-12p40, CD3-delta and connexin 43 (Hori *et al.*, 2004; Kim *et al.*, 2008; McLaughlin *et al.*, 2010).

Anticancer properties of celecoxib are ER-mediated

In therapies of the ER aiming at selective inhibition of secretory chaperone client proteins such as IL-12, overloading of the UPR response into apoptosis is undesirable. Low cytotoxicity and apoptosis observed at 50 μM (Alloza *et al.*, 2006; Tsutsumi *et al.*, 2006) in HEK293 cells suggest that, over the duration of typical experiments (16–24 h), cells can successfully compensate for calcium perturbation through increased chaperone expression via activation of the UPR. This facet of celecoxib presents itself in another therapeutic aspect – that of cancer. The fact that hypoxia and hypoglycaemia are two characteristics of the tumour microenvironment (Scriven *et al.*, 2009) makes it unsurprising that cancer cell survival is strongly linked to the UPR with GRP and UPR chaperones upregulated in numerous cancers (Wang *et al.*, 2005; Daneshmand *et al.*, 2007; Zheng *et al.*, 2008) in order to cope with increased ER stress. Celecoxib has also been shown to inhibit the growth of a number of cancer cell lines in a COX-2 independent manner (Chan *et al.*, 2005; Lou *et al.*, 2006) while the potent COX-2 inhibitor rofecoxib was not (Kulp *et al.*, 2004). The non-coxib analogue DMC has anticancer properties which have been confirmed to be both independent of COX-2 and relying upon ER-mediated UPR induction (Kardosh *et al.*, 2005).

Celecoxib, Anti-inflammatory to cancer drug and back again?

The improved gastrointestinal tolerance but increased cardiovascular risks associated with celecoxib and other COX-2

inhibitors, such as rofecoxib, has been covered in numerous reviews (Frampton and Keating, 2007). The finding that both celecoxib and the non-coxib analogue TFM-C inhibit secretion of pro-inflammatory IL-12, IL-23, and both pro- and anti-inflammatory p80, represents an unexplored ER targeting property pertaining to the original celecoxib, one which may be of applicable translational interest for other diseases. Pending further investigation, the anticipated minimal concentration (micromolar range) needed for TFM-C, DMC or celecoxib to induce COX2-independent cancer cell apoptosis or inhibition of IL-12-type protein secretion *in vivo*, may be reachable in view of data showing peak plasma concentrations in nude mice for DMC or celecoxib of 45 $\mu mol \cdot L^{-1}$ for animals receiving the highest drug dose (Pyrko *et al.*, 2007). Nevertheless, the main translational benefit arising from the TFM-C/IL-12 & IL-23 studies (Alloza *et al.*, 2006; McLaughlin *et al.*, 2010) may reside in the disclosure of a pharmacologically exploitable and therapeutically relevant ER-centric pathway that drives the redirection of the productive folding/assembly of wild-type, oligomeric, disease-promoting cytokines away from secretion towards degradation in the absence of obvious deleterious effects.

Indirect ER targeting via the UPR

Recently, UPR-modifying compounds have been shown to have an indirect regulatory impact on the ER luminal environment. UPR signalling consists of three distinct pathways, IRE1, PERK and ATF6, a detailed review of which is available (Ron and Walter, 2007). Ron and colleagues identified a small molecule, flavonol quercetin, that is capable of substantially inducing the downstream RNase activity of yeast IRE1 through enhanced dimer formation (Wiseman *et al.*, 2010). This indirect targeting of the UPR via IRE1 presents another avenue of ER chaperone modulation, of a similar nature to the calcium-mediated effects observed via TFM-C.

An alternate approach has been brought to market already, that of the 26S proteasome inhibitor bortezomib (Velcade). Inhibition of proteasomal-ERAD leads to accumulation of unfolded proteins in the ER which cannot be cleared. This in turn induces rampant UPR induction and proapoptotic signalling pathways (Obeng *et al.*, 2006). A phase I trial of bortezomib and celecoxib in patients with advanced solid tumors showed promising early results with no dose-limiting toxicity observed (Hayslip *et al.*, 2007). The ER-centric nature of this treatment modality has been shown by the PDIA2 inhibitor bacitracin, which also enhances the ER stress-mediated effect of bortezomib in melanoma cell lines (Lovat *et al.*, 2008).

The ER GRP chaperone complex, the forgotten twin of the cytoplasmic HSP complex

The most drugged cellular chaperone of any type is HSP90. This is due to the discovery that the cytoplasmic HSP90/HSP70 complex interacts with a web of oncogenic proteins ranging from receptor tyrosine kinases to cell cycle proteins. Under normal circumstances, client protein interactions with HSP90 are transient; in contrast, oncogenic mutants are highly dependent on HSP90 in order to retain a conformationally viable active state (Whitesell *et al.*, 1994; Xu *et al.*, 1999). This has lead to great excitement over the ability of HSP90 inhibitors to disable numerous oncogenic proteins through one cellular target. The most highly studied among these is geldanamycin and its analogues 17-allyamino-17-demethoxy-geldanamycin (17AAG) and 17-dimethylamino-ethylamino-17-demethoxygeldanamycin (17DMAG) (Tian *et al.*, 2004) which are undergoing clinical trials in cancer. On many occasions, what is not readily discussed is that HSP90 is not the only cellular target of this class of inhibitor. Geldanamycin also interacts with the N-terminal ATP site of the mitochondrial HSP90 homologue TRAP1 (Felts *et al.*, 2000) and more significantly for this review, with the ER homologue GRP94 (Randow and Seed, 2001). An illustration of the similarities between the GRP94 and HSP90 complex is shown in Figure 4.

While GRP78 recognizes a broad range of peptides (Flynn *et al.*, 1991; Blond-Elguindi *et al.*, 1993b) and calnexin and calreticulin recognize a glycan moiety (Ellgaard and Helenius, 2001), no such recognition moiety has been identified for GRP94. The limited number of GRP94-associating or -dependent client proteins identified thus far are shown in Table 1. The small client population and selectivity, even between family members such as the TLR and integrin family, are perplexing (Randow and Seed, 2001). While it has been noted that heterodimers are more dependent on GRP94 for assembly, this appears not to be strictly so, as the heterodimeric IL-1 receptor and MHC I (Randow and Seed, 2001) are not affected.

GRP94 inhibition and cancer

As previously outlined, GRP94 is present at elevated levels in numerous cancers. Often, and despite target proteins under investigation being proven to interact with both GRP94 and HSP90, the effects of GRP94 inhibition are rarely investigated (Saitoh *et al.*, 2002; Vega and De Maio, 2003; Hsu *et al.*, 2007). GRP94 inhibition does appear to play a key role, with dual inhibition of HSP90 and GRP94 combining to reduce active cell surface levels of transmembrane receptors implicated in cancer. EGFRvIII in the ER shows a concurrent interaction with GRP94, GRP78, HSP90α/β, the HSP70 isoform HSC70 and HSP90 cochaperone Cdc37 (Lavictoire *et al.*, 2003). The global cellular effect of HSP90 family N-terminal ATP pocket inhibitors on transmembrane proteins such as ErbB2 can be broken down into two distinct branches. The first, mediated by HSP90 via the cytoplasmic domain (Chavany *et al.*, 1996), is the ubiquitination and degradation via the 26S proteasome of existing ErbB2. The second, mediated by GRP94, is that newly synthesised ErbB2 becomes unstable and is retained in the ER, with only trace ubiquitination, a significantly reduced half-life, and is present in an immature endo H-sensitive form (Mimnaugh *et al.*, 1996).

Geldanamycin also induces a loss of signalling from the insulin receptor (IR) as measured by lack of IRS-1 activation. In untreated cells, monomeric αβ insulin receptor precursor chains are converted to the final $\alpha_2\beta_2$ tetrameric form. In

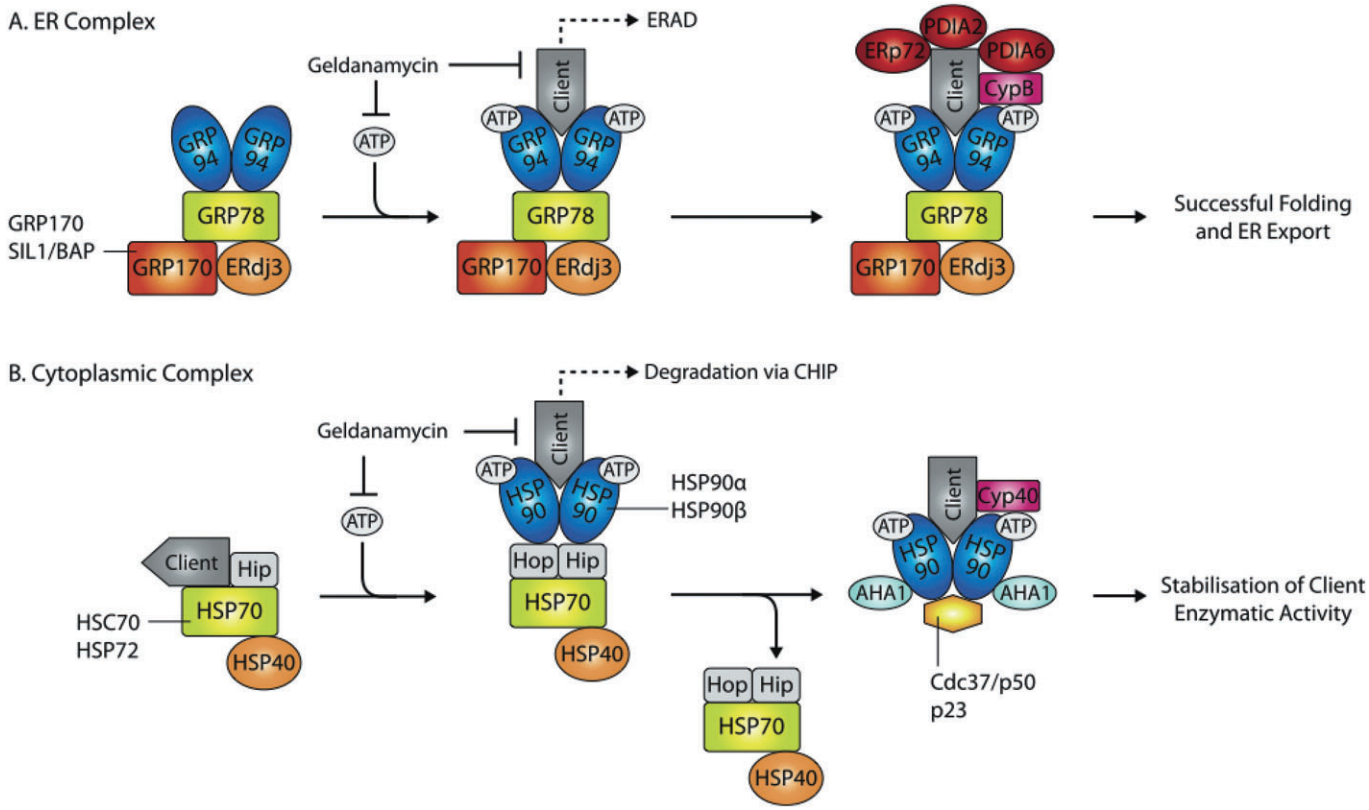

Figure 4

Similarities between GRP94 and HSP90 complexes. (A) The ER GRP complex contains homologues of HSP90, HSP70, HSP40 co-chaperones and peptidylpropylisomerases such as FKBP and immunophilins. GRP94, GRP78 and GRP170 have been shown to be present as a preexisting complex in the absence of folding proteins (the GRP78 HSP40 co-chaperone ERdj3 is also shown throughout to illustrate similarities to the cytoplasmic complex); addition of folding proteins and ATP binding leads to GRP94 chaperone activity and the recruitment of members of the PDI family and the peptidyl-prolyl isomerase cyclophilin B. Inhibition of GRP94 ATP binding by geldanamycin inhibits association with client proteins and leads to proteasomal degradation via ERAD. (B) HSP70s with the HSP40 co-chaperone act to load client proteins onto HSP90 with the interaction mediated by Hip and Hop, which are not present in the ER. HSP70 then leaves the HSP90 complex allowing a mature HSP90 complex to develop containing client protein, immunophilin (in this case Cyclophilin 40 is shown) as well as the HSP90 co-chaperones activator of HSP90 ATPase (AHA1) and client protein stabilising co-chaperones Cdc37/p50 or p23. Thus far, ER homologues of AHA1 or client protein-specific p23 or Cdc37/p50 co-factors have not been identified. This absence may explain the limited number of GRP94 client proteins compared to HSP90. On the other hand, there are many more HSP40 co-chaperones (ERdj1-7) and ATP exchange factors (BAP and GRP170) working in tandem with GRP78 alone and not other HSP70s. Inhibition by geldanamycin prevents client protein association with HSP90 and induces proteasomal degradation via CHIP. It is as yet unknown whether the structure of two GRP94 molecules per single GRP78, based on the cytosolic equivalent, is present in the ER.

geldanamycin-treated cells, insulin receptor assembly progressed only as far as monomer precursor formation with retained precursor chains associating with CNX at elevated levels compared to untreated cells, and rapidly degraded after 2 h (Saitoh *et al.*, 2002). This shows the quintessential paradigm associated with ER retention of plasma membrane receptors due to GRP94 inhibition only.

The insulin receptor has been shown to be elevated in thyroid cancer where it is capable of forming a hybrid receptor complex with IGF-1R (Belfiore *et al.*, 1999), the receptor of insulin-like growth factor (IGF) -I and IGF-II. IGF-I and IGF-II are both single-chain monomers (Humbel, 1990). The lack of free IGFs in serum, with almost all in complex with IGF-binding proteins (Clemmons, 2007) and a half-life for IGF-I of less than 15 min in circulation (Frystyk *et al.*, 1999), is suggestive of an unstable or proteolytically susceptible protein. Use of knockout cells has shown that both IGF-I and IGF-II secretion are dependent on GRP94 (Ostrovsky *et al.*, 2010). IGF-II was still present intracellularly and could be rescued by ectopic expression of GRP94. In wild type cells, 17AAG treatment causes reduced IGF-II-GRP94 interaction and an increase in intracellular IGF-II levels when degradation was blocked by co-treatment with proteasomal inhibitors (Ostrovsky *et al.*, 2009). This strongly links IGF-II to the ERAD-proteasomal pathways upon ER retention, in a similar fashion to that observed upon drug-induced ER retention of IL-12 family dimers by TFM-C (McLaughlin *et al.*, 2010).

In vivo transgenic overexpression of IGF-II in lung epithelium is capable of inducing tumours morphologically similar to human pulmonary adenocarcinoma (Moorehead *et al.*, 2003). In tandem, antisense knockdown of IGF-II reduced *in vitro* proliferation of lung cancer cell lines (Pavelić *et al.*,

Table 1

Known client proteins of GRP94 and techniques utilized in identifying association, inhibition or functional relevance

GRP94 client proteins	IP	GRP94 KO/RNAi	GRP94 drug inhibition
Native proteins			
ADAMTS9 (Koo and Apte, 2010)	+	+	+
Apolipoprotein B (Linnik and Herscovitz, 1998)	+		
Cartilage oligomeric matrix protein (Hecht *et al.*, 2001)	+		
Collagen (Ferreira *et al.*, 1994)	+		
EGF-R (Supino-Rosin *et al.*, 2000)			+
ErbB2 (Chavany *et al.*, 1996)	+		+
Golgi apparatus casein kinase (Brunati *et al.*, 2000)	+		
Ig chains (Melnick *et al.*, 1992; Tramentozzi *et al.*, 2008)	+		
IGF-I (Ostrovsky *et al.*, 2010)		+	
IGF-II (Wanderling *et al.*, 2007; Ostrovsky *et al.*, 2009)	+	+	+
IFN-γ (Vandenbroeck *et al.*, 2006)	+		
IL-12p80 (Alloza *et al.*, 2004; MvLaughlin *et al.*, 2008)	+	+	+
Insulin receptor IRS-1 (Saitoh *et al.*, 2002)			+
Integrins CD11a, CD18, CD49d, α4, β7, αL, β2 (Randow and Seed, 2001; Liu and Li, 2008)		+	
MHC class II (Schaiff *et al.*, 1992)	+		
Bile-salt dependent lipase (Nganga *et al.*, 2000)		+	+
Thrombospondin (Kuznetsov *et al.*, 1997)	+		
Thyroglobulin (Kuznetsov *et al.*, 1997)	+		
TLR1, TLR2, TLR4 (Randow and Seed, 2001; Liu and Li, 2008)	+	+	
TLR9 (Yang *et al.*, 2007)	+	+	
WFS1 (Kakiuchi *et al.*, 2009)	+		
Mutant client proteins			
α-1-antitrypsin (Schmidt and Perlmutter, 2005)	+		
Protein C (Katsumi *et al.*, 1996)	+		
HSV glycoprotein (Ramakrishnan *et al.*, 1995)	+		

2002). Concerted evidence exists in the literature, which indicates that clinical anticancer therapies facilitated by GRP94-mediated reduction of the insulin receptor, IGF-I and II may be possible. IGF-1R inhibitors have been the translational approach of prevalence thus far in targeting insulin/IGF in cancer, including both monoclonal antibody and small molecule antagonists (Gualberto and Pollak, 2009). Translational IGF-II-centric therapies exist along similar lines (Kimura *et al.*, 2010), though are lower in number and significantly less well progressed. Geldanamycin and other GRP94 inhibitors now constitute a third approach for putative IGF-II-targeted anticancer therapies; namely inhibition through ER chaperone-mediated retention.

GRP94 inhibition as an anti-inflammatory

Aside from translational applications of GRP94 inhibition in cancer, immune-related client proteins of GRP94 constitute the largest group identified. A lesser explored aspect of the IGF-I/IGF-II/IGF-1R arm has been its role in autoimmunity pathogenesis, a comprehensive review on which has been recently published (Smith, 2010). The more classical immune-related client proteins of GRP94 are MHC class II (Schaiff *et al.*, 1992), select toll like receptors and integrins (Randow and Seed, 2001), IFN-γ (Vandenbroeck *et al.*, 2006) and the p40 subunit of the IL-12 family of cytokines (Alloza *et al.*, 2004). While secretion or assembly of some of these have been shown to be inhibited by geldanamycin, others have only been shown to interact with GRP94 or to be sensitive to GRP94 knockout (see Table 1). All of these may constitute potential targets for geldanamycin-based inhibition.

Toll-like receptors (TLRs) are cell surface transmembrane proteins of the innate immune system, which mediate recognition of inherently foreign and ubiquitous pathogen-derived molecules. Studies have shown the ability of GRP94 inhibitors to mimic the effects of GRP94 knockout models to induce TLR retention as well as decreased surface presentation (Randow and Seed, 2001; Yang *et al.*, 2007). Inactive mutant GRP94 prevents cell surface presentation of TLR2, TLR4 and TLR9 with the mRNA levels of all three unchanged. Alongside the

integrins CD11a, CD18 and CD49d, all were observed to be retained in the ER. The underlying signal pathways associated with TLR4 were identified to be unaffected by the presence of mutant GRP94, and ectopic expression of GRP94 restored both TLR2 and TLR4 function. Cells lacking GRP94 do not suffer serious disruption of protein folding. Analysis of CRT, GRP78, GRP170, ERp72, ERp57 and PDI shows that none are elevated in GRP94-deficient cells indicating that the loss of GRP94 does not elicit an ER stress response, nor can it be compensated for in the case of assembly of certain TLR chains (Randow and Seed, 2001; Yang *et al.*, 2007).

Coupled to TLR4 is CD14, a glycosylphosphadityl inositol anchored or soluble extracellular pattern recognition protein with the ability to enhance cellular response to LPS through interaction with TLR2 and TLR4 (Finberg and Kurt-Jones, 2006). Putatively in a synergistic effect with TLR2 and TLR4, geldanamycin causes decreased CD14 presentation on the cell surface. Rapid internalisation due to geldanamycin over 2–3 h, independent of new protein production, implicates HSP90 in CD14 internalisation (Vega and De Maio, 2003). As CD14 is present as either a secreted protein or is membrane-attached without a cytoplasmic domain and therefore lacking in any reliance on HSP90 function, this may be as a result of co-internalisation with the TLR4-MD2 complex, which does interact with HSP90 (Triantafilou *et al.*, 2008). As was the case for EGF-R family members, targeting of TLR4 may be susceptible to dual inhibition of HSP90 and GRP94. Due to a substantially smaller client protein population, GRP94 alone may facilitate greater future specificity than dual or HSP90 inhibition alone.

The literature shows that targeting integrins has been approached as an anticancer therapy (Desgrosellier and Cheresh, 2010). Targeting of α4-integrin, a subunit retained upon GRP94 knockout, using monoclonal therapies in a murine model of multiple myeloma, has shown an ability to reduce multiple disease variables (Olson *et al.*, 2005). More interesting in the above work on TLRs and integrins, is the possible treatment synergy in autoimmune disorders between TLR disruption and pro-inflammatory cytokines. While GRP94 inhibition has been shown to have an impact on TLRs and the innate immune system, this immune modulation also extends to the adaptive immune response. The p40 subunit of the IL-12 family of cytokines has been shown to be a client protein of GRP94, with geldanamycin capable of modulating IL-12 family secretion levels (Alloza *et al.*, 2004; MvLaughlin *et al.*, 2008).

TLRs are of relevance as therapeutic targets in a number of scenarios such as exaggerated response to infection (i.e. sepsis), or in chronic autoimmune disorders (Zuany-Amorim *et al.*, 2002). Radicicol and 17AAG have been shown to substantially prolong survival in LPS-challenged sepsis models in mice, with reduced inflammatory markers and capillary leakage while maintaining normal lung function (Chatterjee *et al.*, 2007). HSP90 inhibition was assumed to be the sole mediator of the benefit observed; however GRP94 clearly has a role to play. Indeed, these findings may need to be interpreted to account for loss of responsiveness to TLR ligands, which may have decreased sensitivity to LPS, as has been shown in macrophage-specific GRP94 knockout mice models (Yang *et al.*, 2007). Nevertheless, GRP94 presents itself as a highly interesting and promising therapeutic target in the amelioration of a number of disease states. Both the finding that numerous related proteins such as Fcγ receptor, TNF-R1, connexin-43 (Vega and De Maio, 2003), CD29, CD44, CD45R, CD54, CD121a, CD127 and H-2kb (Randow and Seed, 2001) are all unaffected by GRP94 inhibition, taken together with the low perturbation of the ER environment (Randow and Seed, 2001; Yang *et al.*, 2007), highlight the intriguing selectivity that GRP94 may present for future translational applications (Table 2).

The viability of targeting GRP78

In contrast to the narrow client protein list (Table 1) for GRP94, the GRP78 binding site is thought to be relatively nonspecific with a binding motif on GRP78 client proteins consisting of alternating aromatic/hydrophobic residues which orientate together into the GRP78 binding cleft (Blond-Elguindi *et al.*, 1993a; Rüdiger *et al.*, 1997). While GRP94 appears to be a key potential therapeutic target, GRP78 may be off limits for disruption of specific proteins. The central difference is the overwhelming importance of GRP78 to UPR signalling. GRP78 knockdown results in cells highly primed to ER stress triggered-apoptosis due to its key position as regulator of the UPR (Pyrko *et al.*, 2007; Kardosh *et al.*, 2008). The discovery that the subtilase cytotoxin (subAB) elicits its effects via specific cleavage of GRP78/Bip (Paton *et al.*, 2006) (see Figure 1) has proven that direct targeting of GRP78 can prime cells to ER stress-induced cell death induced by thapsigargin (Backer *et al.*, 2009). This might be advantageous in two facets discussed in this review; as an anticancer agent via a targeted EGF-SubA construct (Backer *et al.*, 2009) or for disruption of secreted proteins. Specifically in the case of SubA, immunoglobulin secretion is blocked due to ER retention on the peptide-binding domain of GRP78, which has been freed from the regulatory ATP-binding domain (Hu *et al.*, 2009).

Direct inhibition of GRP78 *per se* in an attempt to overload the UPR has been described earlier, what of the potential for disruption of select client proteins as for GRP94. With the serious implications associated with GRP78 targeting, its many co-chaperones may represent a workaround capable of inhibiting processing of GRP78 client proteins without compromising GRP78 UPR regulatory function. Thus far, seven human ERdj co-chaperones have been discovered (Otero *et al.*, 2009). These appear to facilitate recruitment of GRP78 to its various discrete functions such as to newly synthesised polypeptides at the Sec61 translocation pore (ERdj2) (Meyer *et al.*, 2000), or to target GRP78 substrates for disulphide bond reduction (ERdj5) (Hosoda *et al.*, 2003, reviewed in Otero *et al.*, 2009). ERdj3 (Shen and Hendershot, 2005) and ERdj6 (Petrova *et al.*, 2008) have been shown to be capable of direct binding to folding proteins, facilitating interaction with GRP78. This may allow targeting of GRP78 client proteins or discrete functions via co-chaperones; however, at this stage so little research exists as to which client proteins may be disrupted to make translational applications impossible to predict. One such example of the promise of this approach has however been identified, that of GRP170.

Much less is known of the true function of GRP170 than of those of GRP78 and GRP94, even though its association

Table 2

List of ER-associated targets, validated client proteins and avenues for applicable translational disease treatment approaches

ER targets	Existing inhibitors	Client proteins	Disease state	References
GRP94	Geldanamycin[¶]	IGF-I / IGF-II	Cancer	(Moorehead *et al.*, 2003; Ostrovsky *et al.*, 2010)
	radicicol	EGF-R / ErbB2	Cancer	(Chavany *et al.*, 1996; Supino-Rosin *et al.*, 2000)
	herbimycin A	Insulin receptor	Cancer	(Belfiore *et al.*, 1999; Saitoh *et al.*, 2002)
		Integrins	Cancer	(Randow and Seed, 2001; Olson *et al.*, 2005)
		Toll-like receptors	Sepsis / Autoimmunity	(Randow and Seed, 2001; Zuany-Amorim *et al.*, 2002)
		IL-12p80	Asthma / Lung inflammation	(MvLaughlin *et al.*, 2008)
Calcium dependent	CE/TFM-C/ DMC[‡]	UPR	Cancer	(Kardosh *et al.*, 2005; Lou *et al.*, 2006)
chaperones		IL-12	MS / RA / Psoriasis / Crohn's Disease	(Alloza *et al.*, 2006)
		IL-23	MS / RA / Psoriasis / Crohn's Disease	(McLaughlin *et al.*, 2010)
		IL-12p80	Asthma / Lung inflammation	(Alloza *et al.*, 2006)
GRP170	None	VEGF	Cancer	(Ozawa *et al.*, 2001)
Glucosidase I and II	CST/dNM[†]	Viral glycoproteins	Viral infection	(Fischl *et al.*, 1994; Chapel *et al.*, 2007)
Ero1	EN460 and QM295	Unknown[#]	Unknown	(Blais *et al.*, 2010)
IRE1	Quercetin	N/A	Cancer	(Wiseman *et al.*, 2010)
Proteasome	Bortezomib[§]	UPR	Cancer	(Hayslip *et al.*, 2007)

MS, multiple sclerosis; RA, rheumatoid arthritis.
[¶]Includes geldanamycin class analogues 17AAG, 17DMAG.
[‡]Indirectly mediated via ER calcium perturbation.
[†]Disrupts glycoprotein interaction and cycling with CRT and CNX.
[#]Ero1 inhibition alters PDI-family member redox regulation; the impact on PDI-family client proteins has yet to be investigated.
[§]Proteasomal inhibition blocks effective ERAD-proteasomal degradation inducing UPR activation.

with GRP78 and GRP94 and role in UPR induction has been known from the initial stages of its discovery (Lin *et al.*, 1993). It has been postulated that GRP170 is simply a GRP78 nucleotide exchange cofactor, similar to BAP (Weitzmann *et al.*, 2006) while its peptide-binding properties classifies it as a 'holdase' only capable of preventing aggregation of hydrophobic regions rather than a bona fide chaperone able to actively refold proteins. A comprehensive review of GRP170 as a putative GRP78 co-chaperone is available (Shaner and Morano, 2007). While GRP170 is capable of peptide-binding in microsomal models (Spee *et al.*, 1999), few well-characterised client proteins exist. These include GP80/clusterin (Bando *et al.*, 2000), IgM, IgG and mutant α-1-antitrypsin (Lin *et al.*, 1993; Schmidt and Perlmutter, 2005), luciferase (Park *et al.*, 2003) and vascular endothelial growth factor (VEGF) (Ozawa *et al.*, 2001).

Irrespective of the true role of GRP170, there is evidence that it occupies a position similar to GRP94 as an abundant co-chaperone with putatively limited general folding functionality and therefore a potential target in inhibiting the assembly of a small number of disease-related client proteins. Of GRP170 client proteins identified so far, vascular endothelial growth factor (VEGF), an angiogenic and vasculogenic mitogen critical in tumour cell invasion and metastasis, is by some distance the most clinically translatable client. GRP170 knock down results in intracellular retention of VEGF (Ozawa *et al.*, 2001) in much the same way as GRP94 knockdown does with IGF-I and –II (Ostrovsky *et al.*, 2010). In the case of GRP170, its role in cancer through elevated levels (Tsukamoto *et al.*, 1998), its absolute requirement for VEGF secretion (Ozawa *et al.*, 2001) and its ATP binding site key for intact functioning (Ikeda *et al.*, 1997), together earmark this chaperone as a candidate of great unexplored translational interest for new potential antiangiogenic therapies.

Targeting the PDI family and ER redox machinery

Of the remaining potential points of therapeutic intervention in the ER, the largest group of chaperones consists of the protein disulphide isomerase family. As proteins with both peptide binding and ATPase sites, members of the PDI family present an opportunity for small molecule inhibitor design. The extensive number of PDI family members, 17 of which have been identified so far (Appenzeller-Herzog and Ellgaard, 2008), is unlikely to represent excessive redundancy but rather specific functional roles which may facilitate targeting of greater selectivity. PDIA2 can dimerise IL-12p40 monomers

to IL-12p80 in cell free assays (Martens *et al.*, 2000). *In vitro*, the PDIA2 inhibitor bacitracin dose-dependently blocks PDIA2-p40 interaction resulting in decreased IL-12p80 but not p40 monomer secretion (Alloza and Vandenbroeck, 2005). While bacitracin has already been outlined previously to enhance the anticancer effect of bortezomib (Lovat *et al.*, 2008), few other therapeutic applications outside combination with bortezomib and *in vitro* inhibition of IL-12 family members exist, and as one of the most broadly active PDI family members PDIA2 may prove an unsuitable therapeutic target in the mould of GRP78. While concerns have been raised over the ability of bacitracin to inhibit PDI (Karala and Ruddock, 2010), in the studies listed, other general inhibitors of thioredoxins were tested in parallel (Alloza and Vandenbroeck, 2005) or ectopic expression of wild type or mutant PDI was carried out alongside bacitracin use to assess the role of PDI (Lovat *et al.*, 2008). Earlier concerns over protease activity in commercial bacitracin preparations have been nullified through purification methods (Rogelj *et al.*, 2000). Of greater relevance is the broad specificity of bacitracin even extending outside the PDI family to the thiol isomerase activity of the integrin αIIbβ3 (Robinson *et al.*, 2006).

ERp29 has been identified to be overexpressed in a number of cancers (Myung *et al.*, 2004; Mkrtchian *et al.*, 2008; Shnyder *et al.*, 2008). Possessing functional protein folding and escort activities (Sargsyan *et al.*, 2002; Ma *et al.*, 2003; Das *et al.*, 2009), what little exists on ERp29 to date points to unexplored therapeutic potential. Elevated levels of ERp29 are linked to increasing infiltration of basal-cell carcinoma (Cheretis *et al.*, 2006), while knockdown of ERp29 may act as a radiosensitiser in rat IEC-6 cells (Bo *et al.*, 2005; Zhang *et al.*, 2008) and reduce tumour size in breast cancer xenografts (Mkrtchian *et al.*, 2008). Of special interest is the prevalence in the literature of ERp29 identification through powerful clinical proteomic studies (Myung *et al.*, 2004; Hoehenwarter *et al.*, 2008). Another poorly characterised PDI member, AGR2/PDIA17, expressed in intestinal epithelial cells, has been shown to form mixed disulphides with and be essential for the secretion of the intestinal mucin glycoprotein MUC2. $AGR2^{-/-}$ mice unable to secrete MUC2 are susceptible to dextran sodium sulphate induced colitis (Park *et al.*, 2009).

Extending beyond the PDI family is that of the ER redox machinery of ERp44 and the ER oxidoreductases (Ero's) (see Anelli *et al.*, 2003). The Ero1α inhibitors EN460 and QM295 prevent $Ero1\alpha_{red}$ from being converted to $Ero1\alpha_{ox}$ with a corresponding inhibition of end-point molecular oxygen depletion. This leads to a pool of $Ero1\alpha_{red}$ which is unable to oxidise thioredoxin. These inhibitors have been shown to have a protective effect against ER stress induced by tunicamycin in $Perk^{-/-}$ hypersensitive *in vitro* models (Blais *et al.*, 2010). As yet, this has not been extended to *in vitro* studies in the context of ER retention of Ero1-dependent cargo proteins but provides evidence of indirect mechanisms with which to target the PDI family machinery of the ER.

Conclusions

In many respects, the body of literature on ERp29, an until recently undiscovered and as yet poorly understood chaperone, can be viewed as a snap-shot of the power of proteomic analysis in directing translational drug research of the ER. This takes the route of a reversed 'bedside-to-bench' approach which firsts seeks to identify biomarkers of disease, rather than the existing serendipitous matching of client proteins to disease states. In the future, identification of disease-related secreted proteins may lead to the generation of an all encompassing 'foldosome,' vis-a-vis a profile of chaperones upon which a given protein is dependent in order to attain a conformationally competent state.

Conversely, AGR2 highlights the potential side-effects of ER-chaperone-targeting, i.e. intracellular retention of therapeutically irrelevant but physiologically important secretory proteins. ER-targeting is likely to exhibit unintended adverse effects. However, this is true of the majority of therapies, as often the most deleterious targets retain other vital physiological functions. Off-target effects may include the general inhibition of CXXC-containing thioredoxins as opposed to specific PDI family members, as well as the inhibition of all three cellular HSP90 homologues by 17-AAG. While a valid concern, off-target effects and lack of potency are an often undesirable property associated with first-in-class small molecules. Further development of second-generation inhibitors, such as NVP-AUY922 in the case of HSP90 (Eccles *et al.*, 2008), can be expected to address many of these concerns.

For the moment, current research into chaperones such as GRP78 and GRP94, and the availability of preexisting small molecules such as celecoxib/TFM-C/DMC and geldanamycin/17AAG with which to target them presents an already significantly progressed translational opportunity (Table 2). Chaperones of the cytoplasm, particularly HSP90, have already provided a translational 'proof of concept' to the viability of such approaches. It remains to be seen whether in the next few years ER chaperones will step out from the shadows and follow their cytoplasmic counterparts into clinical trials and beyond.

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Conflict of interest

The authors state no conflict of interest.

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