Ribosome-inactivating proteins
Potent poisons and molecular tools

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Ribosome-inactivating proteins (RIPs) were first isolated over a century ago and have been shown to be catalytic toxins that irreversibly inactivate protein synthesis. Elucidation of atomic structures and molecular mechanism has revealed these proteins to be a diverse group subdivided into two classes. RIPs have been shown to exhibit RNA *N*-glycosidase activity and depurinate the 28S rRNA of the eukaryotic 60S ribosomal subunit. In this review, we compare archetypal RIP family members with other potent toxins that abolish protein synthesis: the fungal ribotoxins which directly cleave the 28S rRNA and the newly discovered *Burkholderia* lethal factor 1 (BLF1). BLF1 presents additional challenges to the current classification system since, like the ribotoxins, it does not possess RNA *N*-glycosidase activity but does irreversibly inactivate ribosomes. We further discuss whether the RIP classification should be broadened to include toxins achieving irreversible ribosome inactivation with similar turnovers to RIPs, but through different enzymatic mechanisms.

Introduction

Since their discovery decades ago, ribosome-inactivating proteins (RIPs) have been of great scientific interest due to their importance in human health, as both pathogenic agents and therapeutics, but also due to their potential use in biological warfare and bioterrorism. In the past few years, a number of comprehensive reviews have been written on a variety of these subjects.[1-6] RIPs form a family of well-characterized toxins, which specifically and irreversibly inhibit protein synthesis in eukaryotic cells by enzymatically altering the 28S rRNA of the large 60S ribosomal subunit. Most are produced by plants and are thought to represent a defense mechanism against viral or parasitic attackers. Notable examples of plant-derived RIPs include ricin, abrin, and saporins.[2,4] Other RIPs such as the Shiga toxins[3,5] are instead produced as virulence factors by pathogenic bacteria in order to aid their survival and replication in host organisms. Injection, inhalation or ingestion of some of these toxins (e.g., ricin, abrin, and Shiga toxins) can be lethal even in small doses and triggers

irreversible inhibition of host cellular protein synthesis accompanied by acute necrosis of affected tissues and organs. Underlining their potency as pathogenic agents, ricin has been classified as a potential category B biological warfare agent and a likely source of bioterrorism.[7]

Over a hundred RIPs have been isolated from various plants and bacteria with varying degrees of toxicity. These RIPs have been subdivided into two or three broad categories, with the two-category classification currently prevailing.[1,8] In this classification type 1 RIPs are monomeric proteins of approximately 30 kDa which possess RNA *N*-glycosidase enzymatic activity. In contrast type 2 RIPs are composed of an A-chain with RNA *N*-glycosidase activity associated to one or several B-chain(s) of approximately 35 kDa.[5,8-10] The B-subunit is a lectin-like peptide that has strong affinity for sugar moieties displayed on the surface of cells and helps promote translocation through the plasma membrane. As a consequence, type 2 RIPs generally tend to be more toxic than their type 1 counterparts, though this is not always the case since a number of type 2 RIPs (RCA120, Cinnamonin, Ebulin1/r1/r2, Nigrin b, SNA I, SNLRP, IRA b/r) display little or no toxicity despite possessing a lectin-like domain.[11]

The RIP family of toxins was originally described as sharing biochemical properties that lead to irreversible inactivation of eukaryotic protein synthesis by an enzymatic mechanism of action which leads to the abolition of the interaction between the large 60S ribosomal subunit and translation elongation factor 2.[12,13] Toxic RIPs act at low doses because their catalytic activities allow complete inactivation of ribosomes and protein synthesis at a less-than-equimolar ratio to their substrate. RIPs display rRNA *N*-glycosidase activity (EC 3.2.2.22) and depurinate 28S rRNA by cleaving the bond between adenine and ribose in the exposed ype loop of the molecule[14,15] thus preventing recruitment of translation elongation factors and subsequent protein synthesis. Other factors, such as a group of endonucleases isolated from fungi termed ribotoxins also trigger an irreversible inactivation of protein synthesis but through direct cleavage of the 28S rRNA rather than specific depurination. On this basis, they fit the criteria for being RIP family members, but there is controversy as to whether they should be considered true RIPs under the current classification system.[16] In addition to the classification challenges presented by the fungal ribotoxins, a recently characterized bacterial toxin known as *Burkholderia* lethal factor 1 (BLF1) also shares many of the biological properties of RIPs but does not possess RNA

N-glycosidase activity. This factor triggers accumulation of 80S initiating-ribosome species by a different enzymatic mechanism causing a drastic reduction in actively translating polysomes, and an irreversible stalling of translation prior to elongation.[17,18]

In this review, we compare the properties and potential applications of several well-characterized RIPs (ricin/abrin, Shiga toxins, and saporins) with those of the fungal ribotoxins and the bacterial toxin BLF1. In addition, we review the standing of non-RNA *N*-glycosidase ribosome inactivators within the current classification system, and ask whether the definition of a RIP should revert to a broader description which would take into account all enzymatic activities that irreversibly prevent translation elongation from occurring.

Ricin and Abrin (Plant Type 2 RIPs)

Ricin and abrin are among the best-characterized RIP family members and can be extracted from the seeds of *Ricinus communis* (castor bean plant) and *Abrus precatorius* (jequirity pea) respectively.[19] Typical pathological symptoms of exposure to plant type 2 RIPs include abdominal pain, vomiting, diarrhea leading to fluid loss, electrolyte imbalance, and dehydration. Postmortem features include characteristic hemorrhagic intestinal lesions and histology which is consistent with localized cellular apoptosis and tissue necrosis.[20]

There have been other highly toxic type 2 RIP isolated from plants, including Modeccin, Pulchellin, Mistletoe lectin I, and Volkensin, but these will not be covered in this review, which focuses on comparing archetypal members of the RIP family with other potent inhibitors of cellular protein synthesis such as the α-sarcin and BLF1 toxins. The first recorded isolation of ricin was by the German scientist H Stillmark in 1888 during his doctoral work. The same research group, headed by the pioneering toxicologist R Kobert, also identified abrin as being a toxic protein. Early experiments in which the two purified proteins were tested on blood led to their classification as agglutination factors (aka agglutinins) since they induced the clumping of erythrocytes and the precipitation of serum-soluble proteins. Originally this agglutination was thought to be the cause of their toxicity but later work by P Ehrlich in 1891 hinted that this might not be the case. Ehrlich suggested that to be able to work the toxins needed to be fixed in tissue and hypothesized that the protein might consist of a binding region, named "haptophore", and a toxin part, termed "toxophore". When the crystal structures of both proteins were solved his hypothesis was shown to be remarkably close to the truth.[21,22] Both proteins are heterodimers consisting of two disulfide-linked polypeptides, known as the A-chain and B-chain, which have distinct functions.[8-10] The catalytic A-chain resembles Ehrlich's toxophore while the lectin-like B-chain neatly fits the requirements of his haptophore.

Prior to the solving of its structure, ricin was shown to be a potent inhibitor of protein synthesis in intact metazoan cells[23] as well as in cell-free systems.[24,25] Since this inhibition was achieved with concentrations which were sub-stoichiometric to the number of ribosomes present, the mechanism of toxicity was assumed to be enzymatic in nature.[25] Here, the significance of the A-chain becomes apparent since it possesses enzymatic ability in the form of RNA *N*-glycosidase activity. Investigation of this activity revealed that the enzyme specifically acted on an extended loop near the 3′ end of 28S rRNA in the eukaryotic ribosome[14,15] providing a direct link between the function and the observed cellular effects. The glycosidase enzyme depurinates rRNA cleaving the glycosidic bond of a single adenine residue in the exposed loop (A_{4324} in rat liver). This leads to loss of the adenine base but not direct cleavage of the RNA chain. Instead it is thought that the RNA is left susceptible to hydrolysis and may be cleaved by cellular lyases.[26] Subsequently, this mechanism has been found to be the same or very similar in many other RIPs with the shared site of action becoming known as the sarcin–ricin loop. Significantly, this loop is required for recruitment of factors needed for translation elongation,[12] meaning even small disruptions to its structure have severe effects on protein synthesis. Remarkably a single molecule of either ricin or abrin is able to inactivate over a thousand ribosomes per minute,[27,28] effectively leaving cells unable to assemble new ribosomes quickly enough to remain viable. However, as potent as it may be, the presence of an RNA *N*-glycosidase domain alone is not enough to explain the extreme toxicity of ricin and abrin, with LD_{50} = 8.0 μg/kg and LD_{50} = 2.8 μg/kg respectively in mice.[1] To have that level of toxicity they must also have an effective way of crossing the plasma membrane and entering host cells to mediate their harmful effects. For this to occur an RNA *N*-glycosidase domain is not sufficient, a second functional domain is required. This second domain is the B-chain and is the defining feature of plant-derived type 2 RIPs. The B-chain belongs to a family of carbohydrate-binding proteins called lectins.[9,10] These proteins exhibit highly specific binding to sugar moieties within a larger carbohydrate or as part of glycoprotein/glycolipid molecules. In the case of ricin/abrin their lectin-domains exhibit binding specificity for galactose and/or *N*-acetyl-galactosamine or less frequently *N*-acetyl-neuramic acid.[29] It is this property of lectins that confers their haemagglutinating ability, allowing them to recognize carbohydrate groups displayed on the surface of erythrocytes, bind to them, and cause clumping of the blood cells. Most cells are covered in millions of potential binding sites for ricin/abrin in the form of terminal galactose residues on glycoproteins/glycolipids. Once bound, the toxins are absorbed into the cell by endocytosis either via clathrin-dependent[30] or clathrin-independent[31] mechanisms and are directed to early endosomal vesicles. The majority of the toxin molecules are then either transported to lysosomes for proteolytic degradation or recycled to the cell surface.[4] However, around 5% of the toxin is able to find its way into the *trans*-**G**olgi **n**etwork (TGN)[32] and it is from this pool that the active toxin is eventually produced. The importance of this transport to the TGN is underlined by the observation that cells resistant to ricin show impaired endosome-to-Golgi transport at low temperature.[33] Exactly what happens to ricin when it reaches the TGN is not fully understood but it is known that it is moved via retrograde transport through the Golgi stack and into the endoplasmic reticulum (ER). It has been hypothesized that this may be achieved through a number of routes[34] and may be mediated by interactions between the B-chain and galactosylated substrates.[35] Once in the ER lumen
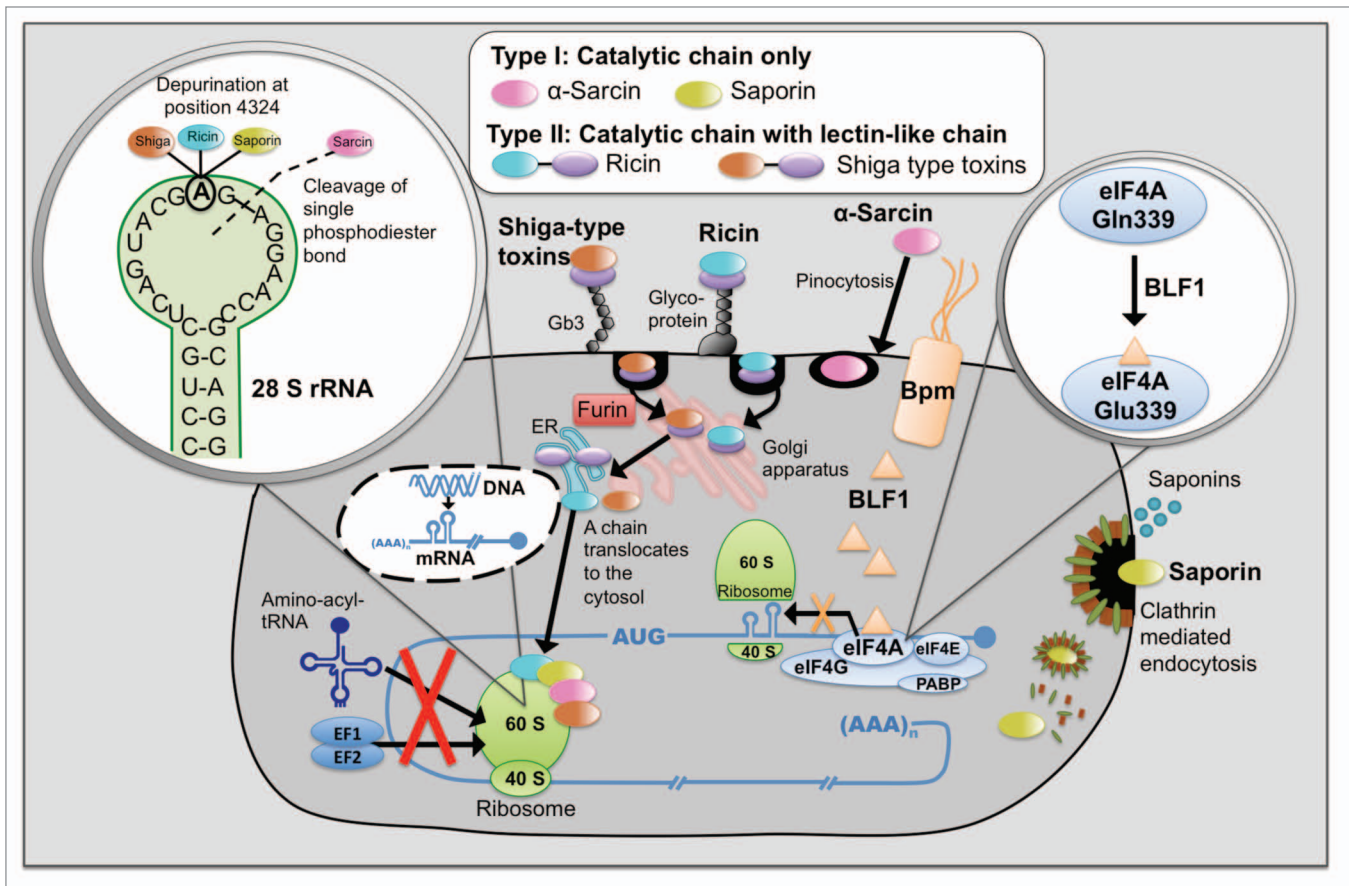
Figure 1. Mechanisms of cellular entry and actions of RIPs. Shiga toxins are delivered into the cell after the B-chain binds globotriaosylceramide (Gb3) to stimulate clathrin-dependent/independent endocytosis. Upon reaching the Golgi the A-chain is cleaved by the protease Furin. Ricin is delivered into the cell after the binding of the lectin-like B chain to glycoproteins/glycolipids and clathrin-dependent/independent endocytosis. After retrograde translocation from the Golgi to the ER the A- and B-chains of ricin and Shiga toxins are separated. Saporin is delivered into the cytoplasm via a clathrin-dependent mechanism of uptake aided by saponins. Shiga toxins, ricin and saporin all inhibit translation using RNA *N*-glycosidase to depurinate 28S rRNA at the same adenine residue (A_{4324}). Alpha-sarcin enters the cell via pinocytosis and cleaves the phosphodiester bond between G_{4325} and A_{4326}. BLF1 is delivered into the cytoplasm via an intra-cellular pathogenic bacterium and deamidates the translation initiation factor eIF4A inactivating its RNA-helicase activity leading to inactivation of initiating ribosomes.

the remaining toxin, or at least the A-chain portion, is then trans-located to the cytoplasm.[36,37] It has been suggested that the A and B chains are separated in the ER through reduction of their disulphide bonds[38] and that this in turn allows the A-chain to be retro-translocated to the cytoplasm through a pathway usu-ally reserved for disposal of misfolded cellular proteins.[39] While a large proportion of toxin directed down this route (known as the ER-associated protein degradation or ERAD pathway) is ubiqui-tinated and degraded by the proteasome upon arrival in the cyto-plasm, a small fraction is able to escape this surveillance and bind to its ribosomal target. It is thought that the differences in toxicity of type 2 RIPs can at least in part be explained by their varying success in avoiding ERAD degradation. A diagram summarizing toxin uptake and action is presented in **Figure 1**.

While the defining feature of both ricin and abrin is their ability to inhibit de novo protein synthesis they also display other properties that contribute to cytotoxicity. For over 20 years there have been reports of RIP-induced apoptosis in affected cells.[40] It was initially assumed that ricin/abrin-induced apoptosis would

be exclusively mediated by the ribotoxic stress response, however in the intervening years a number of different pathways have been implicated.[41] The most prominent examples are the induc-tion of DNA damage through removal of adenine from DNA[42] and activation of the mitochondrial pathway of cell death.[43] Furthermore, it has been demonstrated that both ricin and the Shiga toxins inhibit resolution of H_2O_2-induced DNA lesions, potentially through direct interaction with the repair machin-ery.[44] Interestingly there have also been reports of an apoptotic pathway that is A-chain independent and therefore not connected to the inhibition of protein synthesis.[45] It has been hypothesized that this may involve the B-chain bringing together pro-apop-totic receptors at the cell surface.

Shiga and Shiga-Like Toxins (Bacterial Type 2 RIPs)

While almost all type 2 RIPs have so far been isolated from plant species, the Shiga and Shiga-like toxins are instead pro-duced by gram-negative bacterial species. In the late 19th century

Kiyoshi Shiga, a Japanese physician and bacteriologist, was investigating the cause of dysentery during a large epidemic in which over 90 000 cases were recorded and the mortality rate was as high as 30%.[46] During this epidemic Shiga isolated a gram-negative bacillus bacterium from the stool of patients, which he termed *Bacillus dysenteriae.* This bacterium when fed to dogs led to the onset of diarrhea and toxic factors were found in autolysates of the bacterial cultures. This bacterium and the virulence factor it produces were later confirmed to be one of the major causes of bacillary dysentery and were renamed *Shigella dysenteriae* and Shiga toxin in honor of his discovery. While early work suggested Shiga toxin to be a neurotoxin since it caused limb paralysis when injected into animal models, by the 1970s a relevant gastrointestinal effect of the protein had been established.[47] Soon after it was found that isolates from certain strains of *Escherichia coli* contained a factor that was able to kill Vero cells in vitro.[48] These factors were referred to as verotoxins and the bacteria that produced them were termed **Vero**toxin-producing *E. coli* (VTEC). However, this definition was subsequently altered a few years later when O'Brien and colleagues identified strains of *E. coli* with isolates that contained a toxin related to Shiga[49] that caused hemorrhagic colitis and infantile diarrhea.[50] When it was shown that the verotoxin and Shiga-like toxins could both be neutralized by antibodies to the original Shiga toxin, researchers soon realized they were working with the same strains[51] and led to the strains being reclassified as **S**higa-like **t**oxin-producing *E. coli* (STEC). The fact that remarkably similar toxins were present in two unrelated bacterial species strongly suggested the horizontal transfer of a mobile genetic element. This was proven to be the case when it was found that the Shiga toxins (Stxs) were encoded by genes within the genomes of working or non-functional lamboid bacteriophages.[50] These Stx-phages are able to integrate into the host chromosome and are defined by the presence of the Stx-operon, comprised of the genes necessary for toxin production. Apart from this element, the Stx-phages display a high degree of heterogeneity and mosaicism[52,53] most likely due to recombination events caused by the presence of multiple phages within the same bacterium.[54] Significantly, it has been shown that Stx-phages infect other commensal bacteria in the gut, effectively recruiting them to aid in toxin production.[55,56] This poses problems during outbreaks of Shiga-related illness as symptoms and severity can vary between cases infected with the same strain.

As revealed by X-ray crystallography, all Shiga toxins are of the AB$_5$ protein family[57,58] and are composed of subunits with functions analogous to those of the classic type 2 RIPs identified in plants. A catalytic monomer, termed StxA, is non-covalently associated with 5 surrounding B-fragments that form the B-subunit StxB. Both peptides are synthesized and secreted into the bacterial periplasm where they are assembled into a holotoxin[59] with StxB resembling a doughnut-shaped pentamer into which the carboxyl terminus of StxA sits. In terms of protein sequence there is remarkable similarity between the prototype Shiga toxin (Stx) from *S. dysenteriae* and the major Shiga-like toxins found in STECs (Stx1 and Stx2). Stx and Stx1 are the most similar, sharing identical B-fragments and differing by a single amino acid in the A-subunit.[60,61] Interestingly, Stx2 only shares 56% protein homology with Stx/Stx1 despite having an identical mechanism of action.[62]

As mentioned, the Stx proteins can be thought of as analogous to the plant toxins ricin and abrin and this is reflected in both their function and mechanism of action. As with ricin/abrin the A-subunit provides the RNA *N*-glycosidase activity while the B-subunit displays high affinity for specific cell surface sugar moieties. Remarkably, the RNA *N*-glycosidase activity is identical to that found in ricin/abrin with depurination of the 28S rRNA sarcin–ricin loop occurring at exactly the same adenine residue (A$_{4324}$).[63,64] Again, removal of this adenine prevents binding of elongation factors and associated amino-acyl tRNAs and results in stalling of protein synthesis at the elongation stage.[65,66] Like all classic type 2 RIPs, the Shiga toxins are also able to gain entry into intact cells, and this is mediated by the StxB portion of the assembled protein. Each B-fragment contains three distinctive binding sites for the trisaccharide side chain of glycosphingolipid globotriaosylceramide (Gb3), a glycolipid displayed on the surface of many mammalian cells,[67,68] and binding to this group leads to the internalisation of the toxin by endocytosis. The reliance on Gb3 for Stx protein entry was emphasized by elegant experiments in which Stx resistant cells were made susceptible to the toxin by incorporation of Gb3 into the plasma membrane[69] or where previously susceptible cells were rendered immune through deletion of the Gb3 synthase gene.[70] The only known exception to this Gb3-dependence occurs in porcine models where the Stx2 variant Stx2e exhibits specific affinity for globotetraosylceramide (Gb4).[71,72] In general terms the mechanisms of endocytosis of the Shiga toxins and their transport through the Golgi and ER are very similar to those previously described for ricin/abrin and therefore will not be discussed in detail.[73,74] In common with the diphtheria and *Pseudomonas* toxins[75] Shiga toxins must be cleaved by the membrane-associated protease furin in order to become functional as toxins in the cytoplasm.[76] Furin cleaves StxA at a protease-sensitive loop toward the C-terminus at an alanine moiety between Arg-251 and Met-253 to produce a large catalytic A1 fragment (~27.5 kDa) and a small StxB-associated A2 fragment (~4.5 kDa).[76] The A1 and A2 fragments remain associated with each other through a disulphide link which must be reduced after retrograde transportation to the ER lumen in order to free the catalytic A1 fragment for transfer to the cytoplasm.[77,78] Again, like other type 2 RIPs, it is thought that transport to the cytoplasm is achieved through ERAD since Shiga toxins have been successfully co-immunoprecipitated with chaperone and translocon factors associated with this pathway.[79,80] Significantly, approximately only 4% of molecules make it through all the transportation processing events that allow them to become mature toxins capable of RIP activity in the cytoplasm[81] (**Fig. 1**).

Outside of their RIP function the Shiga toxins activate various signaling pathways within host cells which lead to apoptosis by mechanisms similar to those affected by ricin/abrin[82,83] including those related to DNA damage.[42,44] Activation of the ribotoxic stress response via one of the three major cellular MAPK cascades represents the most commonly activated pathway and happens as a direct consequence of ribosome inactivation.[84] Significantly, activation of this response has also been linked to release of

cytokines that cause upregulation of the Gb3 receptor protein in neighboring cell types, thereby potentially sensitizing them to infection by the toxin.[85] An additional way in which Shiga toxins are thought to induce programmed cell death is through an ER quality control measure known as the unfolded protein response (UPR). This is proposed to be triggered by transient unfolding of the A1 fragment prior to cytoplasmic translocation leading to stimulation of ER stress receptors with a subsequent release of Ca^{2+} ions and activation of cellular caspases.[86] There is also evidence that the purified B-domain of Stx1 is able to stimulate ER stress-independent apoptosis in Burkitt's lymphoma cells, potentially by cross-linking of Gb3 receptors at the cell surface.[87,88]

Saporins (Plant Type 1 RIPs)

So far this review has considered the type 2 RIPs, which, despite being the most extensively studied class only account for a small proportion of all known RIPs.[8] The majority of RIPs discovered so far actually belong to type 1 and are mostly produced in plant species, with preferential distribution within particular families such as *Caryophyllaceae*, *Cucurbitaceae*, and *Euphorbiaceae*.[8] While type 2 RIPs can be extremely toxic due to the presence of lectin-like B-chains and their ability to promote entry into target cells, the type 1 RIPs by comparison are much less harmful. This lack of toxicity is not due to them being poor ribosome inactivators, since they are potent inhibitors of translation in cell free systems.[89] It is instead because they lack an efficient means of entering host cells.[1] In fact type 1 RIPs in plants such as spinach[90] and tomato[2] can be consumed raw without posing any threat to health.

Many type 1 RIPs have been studied extensively, including gelonin, PAP, momordin, and trichosanthin. However, the best characterized and most widely utilized type 1 RIPs are the saporins which can be extracted from roots, leaves and seeds of *Saponaria officinalis*, commonly known as soapwort.[91] In total, nine related RIPs with alkaline pI values have been isolated from soapwort. Saporin-6 is the most abundant and the form most commonly considered the archetypal saporin. The structure of saporin-6 is very similar to that of the A-chain of type 2 RIPs[92] and it displays the universally conserved RNA *N*-glycosidase activity specific for A_{4324} of 28S rRNA.[93,94] In addition saporins are extremely stable when exposed to a number of denaturing conditions.[95] While the lack of a B-chain moiety means that saporins generally show low toxicity in most cells this is not always the case. There is conflicting evidence as to whether saporins can stimulate endocytosis by binding to the surface receptor α_2-macroglobulin[94,96] while a clathrin-dependent mechanism of uptake involving the detergent-like saponins (also produced in soapwort) has been proposed.[97] Once internalized, saporin is trafficked to the cytoplasm through a different route to that of the ricin A-chain utilizing a Golgi-independent mechanism which does not require low pH for membrane translocation[98] (**Fig. 1**). Because of the relative safety in handling saporins and their extreme stability they are extensively used as a therapeutic/research tool when conjugated to other biological molecules that target specific cell types.

Fungal Ribotoxins

Though plant-derived proteins make up the vast majority of proteins that inhibit protein synthesis, a handful have been isolated from fungal species, of which α-sarcin (henceforth referred to as sarcin) is the most famous.[99] Sarcin is a monomeric protein secreted by the fungus *Aspergillus giganteus* that has been shown to inhibit protein synthesis in cell-free systems.[100] More precisely, sarcin belongs to a family of fungal ribotoxins[16,101] with specific RNA endonuclease activity centring on the sarcin–ricin loop of 28S rRNA.[102] Despite targeting the same rRNA structure as the RNA *N*-glycosidase RIPs, sarcin and related ribotoxins destroy the function of ribosomes by a wholly unique mechanism. Instead of depurinating the rRNA through removal of A_{4324}, the phosphodiester bond between G_{4325} and A_{4326} is cleaved.[103] Just like depurination, this alteration disrupts the structure of the sarcin–ricin loop preventing recruitment of elongation factors essential for protein synthesis (**Fig. 1**). Structural studies on multiple ribotoxins revealed that sarcin, RNase T1, and RNase A shared a common catalytic mechanism and belong to the cycling class of ribonucleases.[104] It was later shown that Arg-121 is essential for the RIP activity and cytotoxicity of sarcin since when this residue was replaced (while retaining protein conformation) both were abolished.[105] Because of the disparity in enzymatic function between the ribotoxins and the classic *N*-glycosidase activity of RIPs, there is controversy in the field as to whether ribotoxins should be considered as true RIPs.[16]

Burkholderia Lethal Factor 1 (BLF1)

As described above, sarcin and the other fungal ribotoxins inactivate ribosomes using a mechanism that does not require depurination of the 28S rRNA. The recent discovery of the BLF1 toxin, which like the Shiga toxins before it, originates in pathogenic bacteria, represents a new addition to this group of RNA *N*-glycosidase independent ribosome inactivators.

The intracellular pathogen *Burkholderia pseudomallei* is the causative agent of melioidosis, a chronic and often fatal infectious disease which affects millions people across the world.[106] The bacterium was isolated a century ago[107] but the mechanisms by which it kills and/or remains dormant for decades within infected hosts still remain largely unknown. A breakthrough against melioidosis was made in 2011 when an international team of researchers lead by the Wilson and Rice groups from the University of Sheffield characterized the first lethal cytotoxic factor from *B. pseudomallei* which irreversibly inactivates host protein synthesis.[17] This protein is thought to be the major cause of *B. pseudomallei* pathogenesis, though it is possible that other toxins also contribute to cell death/tissue necrosis given that the bacterium has a well-stocked arsenal of virulence factors.[108]

This lethal toxin was found to be encoded by a gene of unknown function systematically named *BPSL1549* after sequencing of the *B. pseudomallei* genome in 2004.[108] BPSL1549 toxin was subsequently renamed BLF1 for *Burkholderia* lethal factor 1.[17,18,109] Comparison of the proteomes of *B. pseudomallei* and the non-pathogenic but related strain *Burkholderia thailandensis* revealed

that 14 uncharacterized protein biomarkers were expressed in the extracts of the pathogenic strain.[110] A structural program aimed at investigating some of these proteins led to the determination of the crystal structure of BPSL1549/BLF1 by the Rice group.[17] Injection of the recombinant protein intramuscularly or through the intraperitoneal route kills exposed mice with 10 μg BLF1 plus adjuvant, or 100 μg BLF1 alone while transfection of BLF1 also kills mammalian cells lines, as well as macrophages. Potent cytotoxic effects are achieved with a concentration as low as 2.5×10^{-7} M.[17] This is similar to concentrations described for RNA depuration by ricin which has a K_M of $1–2 \times 10^{-7}$ M.[28] BLF1 was shown to be a deamidase enzyme which specifically deamidates Gln-339 of the eukaryotic translation initiation factor eIF4A inactivating its RNA-helicase activity.[17] This RNA-helicase activity is thought to be required for unwinding secondary structures in the 5′ untranslated regions of circularized mRNA prior to the assembly of the large and small ribosome subunits and initiation of translation. The eIF4a factor has been shown to be essential for the recruitment of the 40S ribosomal subunit (and other initiation factors) to capped mRNA and remains associated to initiating ribosomal complexes during scanning prior to translation elongation.[111-114] Significantly, while the action of BLF1 does not directly damage the ribosome, it is important to note that the BLF1-modified eIF4A factor cannot be recycled for new rounds of translation initiation as pre-initiating 80S ribosomal complexes are irreversibly stalled on mRNA molecules rendering them lost. This leads to a pronounced accumulation of stalled 80S ribosomes and a concomitant decrease in the number of polysomes measured in sucrose sedimentation experiments. Indeed, in cell-free systems affected by BLF1 exogenous recombinant eIF4A cannot be incorporated into the stalled ribosomes, further emphasizing the irreversible nature of the ribosome inactivation and the dominant negative effect of the BLF1-modified eIF4A on ribosome function.[17]

The catalytic turnover of BLF1 was determined to be around 700 substrate molecules per minute,[17] a rate similar to the rRNA depuration catalyzed by ricin,[27,28] suggesting that the high turnover of BLF1-dependant inhibition of protein synthesis leads to cell death when all functioning ribosomes in a cell are inactivated by modified eIF4A[18,109] (**Fig. 1**).

RIPs as Potential Biological Weapons and Criminal Agents

In 1997, the US government designated several RIPs as "select agents" with the "potential to pose a severe threat to public health and safety". Since then, *Burkholderia pseudomallei*, the related bacterium *Burkholderia mallei,* and several toxins, including ricin, were classified as category B biological warfare agents.[7] Category B weapons are described as "agents with some potential for large-scale dissemination with resultant illness, but" which "generally cause less illness and death" than category A agents such as anthrax and botulinum.

Ricin was reported to be investigated by the US biological weapon program during World War I and II[115,116] as well as Canada during World War II[116] for utilization as weaponized toxic dust or coated onto bullets and anti-personnel mines, though it

was never used in combat. This seems in part due to the fact that disease-producing spores of other biological agents such as *Bacillus anthracis* or *Clostridium botulinum* persist much longer in the environment than a purified protein such as ricin. Furthermore, it was assessed that several tons of ricin powder would be required to target urban populations making ricin unsuitable as a biological weapon of mass destruction.[117] However, ricin continues to remain in the public consciousness mainly because of its potential for usage by terrorists/criminals in assassination plots or acts of terror, with this fear coming from the fact that it is relatively simple to isolate from an easily accessible source (castor bean plant). Famously, in 1978, ricin was used in the murder of a London-based Bulgarian dissident, Georgi Markov. Markov was a critic of the communist regime in Bulgaria and it is commonly accepted that the Bulgarian secret service assassinated him using a ricin capsule fired from the tip of an umbrella using compressed gas.[117] Since then, ricin has continued to be used or attempted to be used for murder/intimidation in a number of plots, the latest high-profile incident being a ricin-laced letter sent to US president Barack Obama in April 2013. Because of these continuing concerns regarding the potential for ricin attacks significant efforts have been made to prepare for such events and contingencies have been put in place.[118] This includes the development of effective vaccines such as RiVax™ from the biopharmaceutical company Soligenix Inc., which can immunize mice, rabbits, and humans against ricin exposure.[119]

The BLF1-producing pathogen, *B. pseudomallei*, easily grows outside a laboratory in most climates making it a prime candidate for utilization by terrorists.[120] It can survive in distilled water for a number of years[121] and infects almost all mammals[122] and tomato plants[123] raising concerns about its ease of storage/dissemination and the potential for long-term environmental contamination if used in an attack. No vaccine has yet been formulated despite many attempts.[124] Precedence for the use of *B. pseudomallei* as a bio-weapon comes from the historical use of the closely related bacterium *B. mallei*. This bacteria is the causative agent of "glanders", an equine disease contagious to humans that is very similar to melioidosis.[125,126] It was the first biological weapon used during the American Civil War,[127] and was utilized in World War I by the German army to deliberately contaminate livestock and humans.[128,129] It has also been reported that the Soviet biological weapons program conducted field tests with *B. mallei* which inadvertently killed some of the researchers, and that the bacterium may have been used against the Mujahideen during the Afghanistan War in the late 1970s.[130]

Research and Therapeutic Applications

RIP family members have several practical applications ranging from benefits to agriculture to biomedical therapies as described recently in a review by F Stirpe.[6] RIPs not only constitute potent molecular tools for studying translation but also have potential applications in the treatment of human disease since several inhibitors of protein synthesis have been used as anti-cancer agents including inhibitors of eIF4A.[131,132] Since BLF1 is a potent inhibitor of eIF4A and exhibits high levels of cytotoxicity, it also represents a suitable candidate for investigation of anti-cancer

properties.[18,109] Immunotoxins built by conjugating an antibody recognizing a cancer cell-specific epitope to the A-chain of ricin seemed to be promising therapeutics following in vitro studies in tumor cell lines[133] but have unfortunately shown less efficacy in vivo in mice or humans.[134] In contrast, saporin-based immunotoxins have shown potent in vitro and in vivo anti-cancer activity against prostate cancer.[135] Substance-P-conjugated saporins (SP-SAP) have also been developed as selective destroyers of neurons involved in pain transmission and may provide a method of treating pain in patients with chronic illnesses.[136] Other type I and II RIPs from plants have shown antitumor activity both in vivo and in vitro on cancers from various tissues.[4] However, current challenges involve finding specific and efficient methods of drug delivery as well as overcoming the instability of RIPs in circulating plasma. Chemical conjugation of RIPs to water-soluble polyethylene glycol polymers, which protect against proteolytic cleavage, significantly improves their half-lives while also reducing their immunogenicity.[137] Another issue with the use of RIP-containing immunotoxins is the high immune response they trigger in treated subjects due to them being recognized as foreign proteins. Repeat doses lead to development of immunological memory which severely decreases the half-life, and therefore effectiveness, of the immunotoxins over time.[6]

RIPs have also served as molecular tools to selectively kill specific cell types in order to study their physiological or behavioral relevance. For example, a saporin-based immunotoxin was generated to target and kill rat cholinergic neurons in the basal forebrain to study their function in cognition and behavioral comportment.[138] This in turn led to the discovery of an animal model mimicking Alzheimer disease for drug screening.[138] The potential biotechnological and medical uses of saporin can be attributed to its unusually high resistance to proteolysis and denaturation in solutions containing up to 4 M urea or guanidine[95] as well as its relative safety in its unmodified form.

Concluding Remarks

RIPs can traditionally be sub-divided into two distinct classes based on their structure. Type 2 RIPs, which include the most potent toxins in the RIP family, are heterodimers comprising a catalytic RNA N-glycosidase A-domain linked by disulphide bonds to a lectin-like B-domain that promotes entry into target cells. Type 1 RIPs on the other hand consist only of the A-domain and are generally less toxic as a result. Both classes inactivate ribosomes using a common mechanism in which the RNA N-glycosidase domain depurinates a specific adenine (A_{4324}) within the sarcin–ricin loop of the 28S rRNA molecule leading to structural disruption and subsequent failure to recruit translation elongation factors. However, there are other proteins that can achieve the same end result as classical RIPs without utilizing an RNA N-glycosidase-based mechanism. The fungal ribotoxins, including prominent members such as sarcin, RNase T1, and RNase A, are able to irreversibly inactivate protein synthesis by directly cleaving the phosphodiester bond between G_{4325} and A_{4326} of the sarcin–ricin loop. Just like depurination this alters the secondary structure and prevents translation elongation from occurring. In common with the ribotoxins, the newly identified bacterial toxin BLF1 is also able to affect an irreversible inactivation of protein synthesis using an enzymatic mechanism which does not rely on RNA N-glycosidase activity. Instead of modifying rRNA, BLF1 functions as a deamidase enzyme, specifically acting on the translation initiation factor eIF4A and causing a Gln to Glu deamidation which abolishes its RNA helicase activity. Ablation of this activity prevents eIF4A functioning correctly and causes the modified eIF4A to act as a non-recyclable dominant negative factor when associated with 80S-initiating ribosomes. This leads to accumulation of 80S initiating-ribosome species and prevention of translation elongation, leading to an eventual inactivation of cellular protein synthesis when all active ribosomes have been stalled.

Given their disparate catalytic functions the ribotoxins and BLF1 unsurprisingly show no sequence or structural similarity to classical type 1 or 2 RIPs (**Fig. 2**). However, the cytotoxicity of BLF1 is comparable to that conferred by the highly toxic type 2 RIPs such as ricin or abrin despite being only composed of a single catalytic polypeptide chain. BLF1 also lacks the lectin-like domain required for efficient target cell entry. However, it does not need to be able to provide its passage into host cytoplasm to achieve its high toxicity as the bacterium that produces BLF1, *B. pseudomallei*, is an intracellular pathogen.

As noted earlier, ribotoxins and BLF1 are able to impose an irreversible inhibition of protein synthesis where active elongation no longer occurs. This would place them within early definitions of RIPs which were described as proteins that could irreversibly inactivate eukaryotic ribosomes in a catalytic fashion preventing recruitment of elongation factor(s).[12,13] More recently, the description of RIPs has become restricted to proteins which exhibit the aforementioned properties but also have RNA N-glycosidase activity and has led to inconsistencies regarding the status of proteins such as the ribotoxins and now BLF1. With this in mind we ask whether a return to more inclusive description of RIPs would be beneficial. It is clear that the ribotoxins and BLF1 do not belong within the classical RIP family, but because the cellular consequences of their activities are identical to those of bona fide RIPs (i.e., a block of protein synthesis), it seems appropriate that they be more formally recognized as unconventional members of the RIP family. It is also very likely that more members of both the ribotoxin and deamidase classes of ribosome inactivators will be identified in the future. This is particularly relevant to the deamidase class since *B. pseudomallei* has been shown to have a remarkably fluid genome,[108] suggesting that BLF1 and a number of its other virulence factors may have been acquired horizontally through conjugation and/or transduction meaning there are likely to be similar toxins waiting be discovered in other bacterial species.

In conclusion, RIPs could be described as any protein factor which irreversibly inactivates protein synthesis in an enzymatic manner thereby preventing translation elongation from occurring. This would encompass all the proteins described in this review and be broad enough to accommodate novel toxins and/or enzymatic mechanisms which achieve a similar cellular outcome. The two-division system for classical RNA N-glycosidase
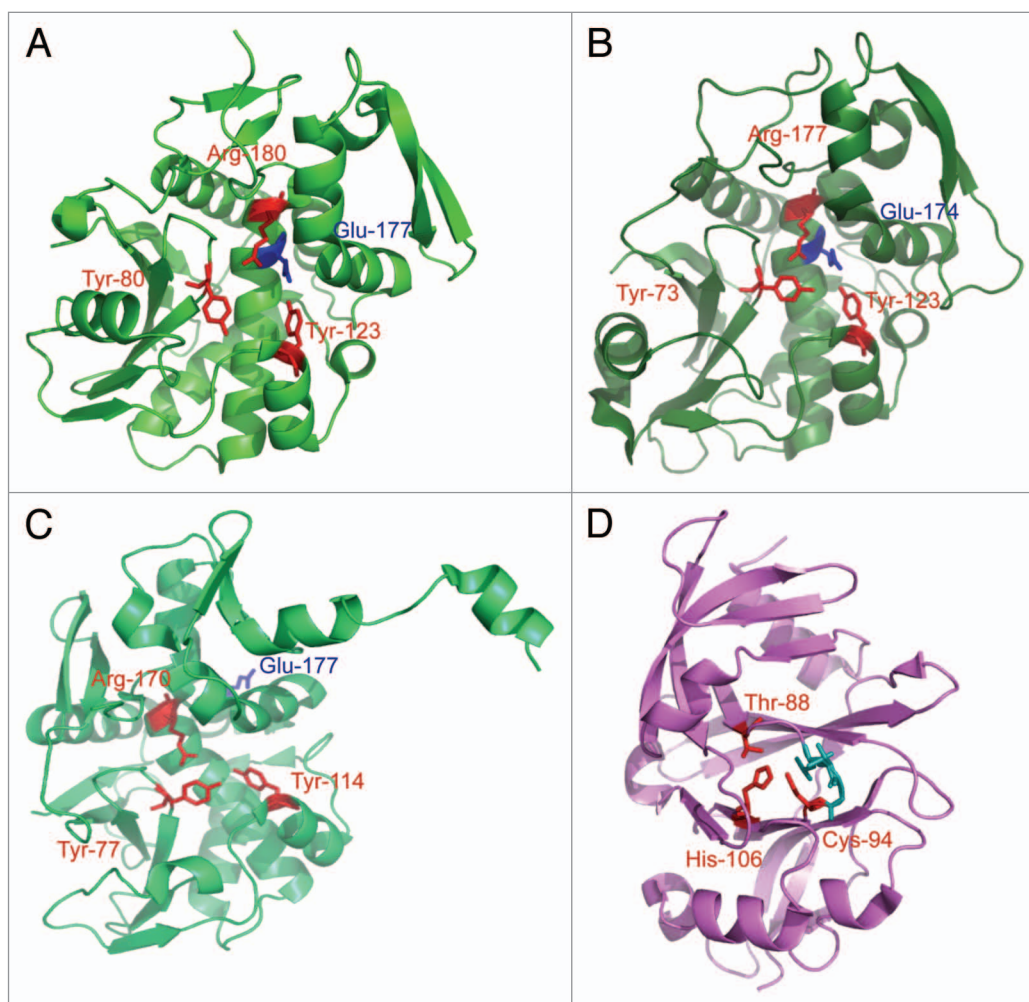
Figure 2. Ribbon representations of the X-ray crystallography structures of type 1/2 RIPs and BLF1. Ricin, saporin, and Shiga toxin Stx2 have *N*-glycosidase activity that cleaves the 28S rRNA while BLF1 catalyzes the deamidation of the eIF4A initiation translation factor. (**A**) Structure of Ricin A-chain in complex with the cyclic tetranucleotide inhibitor; PDB ID code 3HIO.[139] (**B**) Structure of saporin in complex with the cyclic tetranucleotide inhibitor; PDB ID code 3HIW.[139] (**C**) Structure of Shiga-like Stx2 A-chain from *Escherichia coli* in complex with adenine; PDB ID code 2GA4.[140] Red-labeled residues Tyr-80, Tyr-123, and Arg-180 in the active site of ricin A-chain are equivalent in saporin and Stx2 (Tyr-73, Tyr-123, Arg-177 and Tyr-77, Tyr-114, Arg-170, respectively).[92,139-141] The catalytic water molecule and crystallized inhibitors within the active sites are not represented for improving clarity. In contrast, Glu-177 of Stx2 is not conserved with the Glu-177/174 in the active sites of ricin and saporin respectively. Glutamate residues are labeled in blue. (**D**) Structure of BLF1, PDB ID code 3TU8.[17] The primary sequence Leu-91 Ser-92 Gly-93 is (cyan) is conserved in the carboxyl-terminal domain of CNF1 along with Cys-94. Residues forming the catalytic triad of BLF1 (Thr-88, Cys-94, and His-106) are shown in red. Both the tertiary structure and catalytic site of BLF1 are different from type 1 and 2 RIPs represented in (**A–C**).

RIPs could remain while an extra provision could be created for unconventional RIPs which catalyze ribosome inactivation in an RNA *N*-glycosidase-independent manner.

Disclosure of Potential Conflicts of Interest

The authors declare that no conflict of interest exists.

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