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Construction of sea anemone cytolysin-based immunotoxins for selective killing of cancer cells

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Sumario. Las inmunotoxinas (ITs) son proteínas quiméricas constituidas por una toxina y un componente guía que dirige su acción hacia un tipo de célula que se desea eliminar. Las toxinas más utilizadas en la construcción de las ITs afectan la síntesis proteica de la célula blanco. Sin embargo, la citotoxicidad de los inmunoconjugados construidos a partir de esta clase de toxina depende de la internalización de la entidad tóxica hacia el citosol, a lo que se suma que la mayoría de estas ITs son transportadas hacia los lisosomas donde son degradadas. En este sentido las ITs que utilizan como componente tóxico las citolisinas de anémonas de mar (actinoporinas), constituyen una alternativa con ventajas potenciales, pues su mecanismo de acción es a través de la formación de poros en la membrana. Sin embargo, la actividad inespecífica asociada a la toxina ha sido un factor común en todas las construcciones obtenidas hasta el momento con actinoporinas. Una posibilidad de superar esta limitación es la construcción de ITs inactivas con un mecanismo de activación que responda a un estímulo biológico. En particular las citolisinas pueden activarse por la acción de proteinasas asociadas a tumores. En este trabajo se discuten los avances en el diseño de ITs basadas en actinoporinas activables por metaloproteasas de matriz (MMP) así como las perspectivas de su uso como parte de *cocktails* quimioterapéuticos. Se discuten además las ventajas potenciales de emplear solamente la región aminoterminal de las actinoporinas en la construcción de ITs.

Abstract. Sea anemone cytolysins based Immunotoxins (ITs) constitute an attractive alternative for construction of IT for selective killing cancer cells. In fact the pore-forming cytolysins from sea anemones, actinoporins, are one of the most potent groups of pore-forming proteins in nature. The requirement of internalization and translocation to the cytosol and the degradation of most of the IT routed to the lysosomes after internalization would be some of the critical factors determining the cytotoxicity of the classical ITs that would be overcome in such constructions. The results obtained using as toxic moiety actinoporins have supported the feasibility of directing these cytolysins to the surface of either cancer cells or even the parasite *Giardia duodenalis*. However the main problem of the IT constructed in such fashion is the lack of the specificity associated with the toxin moiety, a common problem of most membrane-acting ITs. An approach designed to overcome this limitation is the production of ITs using PFTs activated by tumour-associated proteases. Currently, the construction of an MMP-activated IT based on an actinoporin is in development. The alternative of using actinoporin based ITs as components of chemotherapeutic cocktails and the future prospects of employing only the N-terminal region of actinoporins for construction of IT are discussed.

Keywords: Nanotechnology biomedical applications, 87.85.Qr, 87.85.Rs, 81.16.-c

1 Introduction

Immunotoxins (ITs) are chimeric molecules in which a cell binding ligand, such as a monoclonal antibody (mAb) or a growth factor are coupled to a killer toxin in order to address its activity towards a specific undesired cell. The target can be tumour cells, virally infected

cells, subsets of normal cells or parasite cells. In fact the use of ITs in cancer therapy, graft-vs-host disease, autoimmune diseases, and AIDS has been ongoing for the past three decades. Most of the ITs currently in use or under investigation contain a toxic component, which acts intracellularly to inactivate protein synthesis. Once in the cytosol, a single molecule is capable of killing a cell, making ITs some of the most potent known killing agents. Among the most active clinically are those targeted to tumours, specifically those directed against haematological tumours. Obstacles to successful treatment of solid tumours include poor penetration into tumour masses, toxicity and the immune response to the toxin component. Exploring other classes of toxin to create new ITs is underway to improve the treatment of cancer. Relatively few membrane-acting toxins have been investigated for their use in immunoconjugates, in spite of some inherent advantages to their use.

Pore-forming cytolysins from sea anemones, actinoporins, are one of the most potent groups of pore-forming toxins (PFTs), and they constitute an attractive target for exploitation in the construction of IT. In this review we summarize the main results obtained in the use of sea anemone PFTs in the construction of ITs. We also discuss the biochemical and crystallographic studies that have provided new information on the structure and function of toxins essential to improve the design and construction of such hybrid molecules. In particular, recent elucidation of their crystal structures, advances in the knowledge of the structure and function relationship as well as cloning and expression of these molecules have significantly contributed to the recent progress in IT design.

2 Biochemistry of immunotoxins

***2.1 Targeting moiety*,** The targeting agents currently used to construct ITs are mAbs, growth factors and cytokines but the first ones are the most frequently used. In the case of tumour cells, mAbs that recognize differentiation antigens (Ags) on normal cells are often used because unique tumour associated Ags have not been identified for most human tumours. Since tumour cells often express higher levels of these differentiation Ags, the tumour cells may be preferentially killed. Monoclonal antibodies have been used in their natural, fragmented, chemically modified, or recombinant forms[1]. Basic antibody structure and the location of functional domains are provided in figure 1. The first generation ITs were constructed via chemical linkage (usually a disulfide bond) between toxins and mAbs. In the second generation appeared ITs constructs made with Fab fragments of antibodies (figure 1). The Fab constructs were smaller and potentially enabled greater tumour penetration and a more uniform final product. Third-generation ITs and related growth factor and cytokine fusion proteins are made by recombinant DNA techniques and as a group are called recombinant toxins; those containing Fv portions of antibodies (figure 1) are called recombinant ITs. The two chains of Fv are held together either by a flexible peptide linker (single-chain ITs or by a novel disulfide bond). Recombinant ITs are homogeneous and relatively inexpensive to produce.

2.2 Toxin Moiety

2.2.1 Classical Immunotoxins inhibit protein synthesis . Many protein toxins have been used to make ITs and excellent reviews are available[1-3]. Nearly all protein toxins work by enzymatically inhibiting protein synthesis. The most common toxic moieties used in ITs are derived from either bacteria [e.g. *Pseudomonas* exotoxin (PE) or diphtheria toxin (DT)], or plants (e.g. ricin or abrin). Both types of toxin kill cells by inhibiting protein synthesis, PE and DT inactivate elongation factor 2 (EF-2) and ricin and abrin inactivate the EF-2 binding site on the 28S ribosomal subunit.

These toxin moieties require internalization and translocation to the cytosol to achieve the cytotoxic effect. Once internalized, most of the IT is routed to the lysosomes, where the toxin is degraded[2,4] and this is one of the critical factors determining the cytotoxicity of the classical ITs. However, encouraging clinical trials have been carried out both in haematological malignancies, where the ITs can rapidly reach the tumour cells, as well as in solid tumours, where tumour entry is limited. An important fact is that, Denileukin diftitox, an IT containing human interleukin-2 and truncated DT, has been approved by the US Food and Drug Administration for treatment of cutaneus T-cell lymphoma[5,6] representing the first IT approved for use in humans.

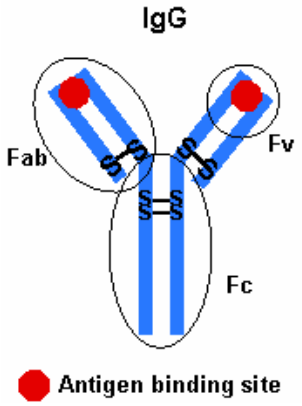

Figure 1. Antibody structure. IgG molecules are divided into functional domains. The main divisions are Fc and Fab. The Fc interacts with cellular Fc receptors and complement; the Fab fragment contains the Ag binding site. The Fab fragment is further divided into the Fv fragment, the smallest fragment that retains Ag binding via contacts with both the heavy and light chains. The two chains of the Fab fragment are held together.

2.2.2 Membrane-acting immunotoxins. The use of a killing mechanism that does not need internalization could be one of the advantages of using membrane-acting toxins as IT toxic components. Despite their potential, relatively few membrane-acting toxins have been investigated for use in immunoconjugates. In some instances, however, membrane-damaging toxins of different origins have been used to build such chimeras. For example, cytolysins from sea anemones[7-12], from the snake *Naja naja siamensis*[13] and melittin, the cytolytic

peptide from bee venom[14] as well as toxins derived from plants (*Pyrularia* thionin[15]) or from bacteria (*Bacillus thuringiensis* δ-endotoxin[16] and *Clostridium perfringens*[17]) have been reported in the literature.

By selectively increasing target cell permeability, immunoconjugates based on membrane-acting toxins could theoretically bypass the major limitation of traditional ITs, namely their requirement of internalization and translocation to the cytosol. In addition, this type of immunoconjugate could strengthen the effects of conventional ITs, other chemotherapeutic agents and common anti-cancer drugs by facilitating their access into the cytosol. By disturbing the plasma membrane, they might reverse the effects of multi-drug resistance. If applied to treat solid tumours, inner cells could be exposed to chemotherapy following lysis of cells located nearer the vasculature[18].

2.2.2.1 Sea anemone cytolysins as components of immunotoxins. The first isolated class of sea anemone cytolysins was collectively called actinoporins[19]. They comprise a group of approximately 20 kDa proteins, lacking cysteine residues whose activity depends on the presence of sphingomyelin (SM) in the membrane. They form pores in natural and model lipid membrane of approximately 2 nm in diameter[20-22]. The formation of transmembrane pores disrupts ion gradients, which leads to osmotic swelling and ultimately to cell death. Actinoporins are extremely cytotoxic and lytic to a variety of cells and their vesicular organelles. The cytotoxic activity of actinoporins on different cell types and cell preparations ranges from 10^{-11} to 10^{-7} M[23]. Taking into consideration this cytotoxic activity actinoporins have been used for construction of ITs. Previous reviews about the use of sea anemone cytolysins as toxic components of immunotoxins are available[23,24]. The cytotoxic activity of the actinoporin based ITs, expressed as mean concentration required for 50 % reduction of viability (C50), is shown in table I and ranges from 10^{-10} to 10^{-8} M.

The first attempt to use a membrane-acting toxin for the construction of an immunoconjugate involved a haemolytic fraction from the sea anemone *Stichodactyla* (formerly *Stoichactis*) *helianthus*. Avila *et al.* first linked the toxin to an mAb that recognizes a specific Ag expressed on immature T-lymphocytes (IOR-T6)[7] and later on the toxin was bonded to an mAb directed against the carcinoembryonic Ag (CEA)[8]. In both cases the cytotoxic activity of the hybrid molecules toward cells expressing the Ag was quasi or totally reversed by the co-addition of an equivalent amount or an excess of the corresponding free antibodies, which saturated the binding sites and prevented the action of the ITs. This result indicated that the hybrid molecule obtained bound the tumour cells through the mAb.

More recently, the same haemolytic fraction was linked to IOR egf/r3, a mAb against the human epidermal growth factor receptor[9]. In this case the cytotoxic activity of the hybrid molecule was also reversed by the co-addition of an excess of free mAb. In addition the differential toxicity observed for this IT against cells expressing different levels of epidermal growth factor receptor was not shown for the free toxin. These two results indicated that the toxicity of the conjugate was mediated by the mAb.

On the other hand, sticholysin I (StI), one of the two isoforms of the cytolysins isolated from the sea anemone *Stichodactyla helianthus*, was linked to an mAb (IOR C5) recognizing the colon tumour-associated Ag IOR C2[10]. The study of the binding capacity of this conjugate to the SW948 colon tumour cell line demonstrated that the hybrid molecule bound the tumour cells preferentially through the mAb rather than by the cytolysin binding region. In agreement the conjugate showed higher specificity of the toxic effect toward the colon tumour cell line than the free cytolysin since the ratio between its cytotoxic and haemolytic activities was approximately seven-fold higher than the ratio found for the free toxin.

EqtII has been conjugated to transferrin (Tfn), a major regulator of cellular growth and a potent mitogen for a variety of tumours. The resulting conjugate showed some specificity of the toxic effect toward tumour cells *in vitro*; however, the hybrid molecule retained unspecific activity due to the fact that Tfn-bound toxin partially retained its original ability to bind to the cell membrane directly[11]. In this work the cytotoxic activity of the conjugate was inhibited three-fold by the presence of an excess of free ferric Tfn, suggesting that it was, at least in part dependent on the expression of the Tfn receptor. However, the residual unspecific activity of the conjugate was still relatively high.

While ITs are best characterized and promising as anti-cancer tools, other uses have also been explored. Specific targeting of the parasitic protozoan *Giardia lamblia* (syn. *duodenalis)* was achieved using the high affinity biotin-avidin system (figure 2A) to concentrate a biotinylated form of a mutant of EqtII (EqtII A179C) on the parasite surface pre-treated with a primary anti-lamblia antibody and a secondary Avidin-conjugated antibody[25]. Such treatment increased specificity of targeting the parasite cells four-fold with respect to those that did not receive the primary mAb, but were otherwise treated in the same way.

A similar approach was carried out by Potrich *et al.*[12] who used the mAb against the Tfn receptor OKT9 and three single cysteine mutants of EqtII (K20C, R126C and A179C) to target the human malignant melanoma cell line A375. The targeting complex enhanced the cytotoxic activity of the mutants to the targeted A375 cells with a two- to three-fold higher specificity factor.

The biotin-avidin method is quite advantageous because it relies on very simple chemistry to create conjugates that can target different cell types. In fact, simply by using a library of primary antibodies, it is possible to selectively target many different individual cell types. Another possibility is to target an individual cell type with cocktails of two or more ITs recognizing different Ags. This alternative can be very useful since it has

been demonstrated in several animal tumour models that the use of a single type of IT fails to provide a complete cure due to the survival and subsequent outgrowth of IT-resistant tumour cells[2]. These cells may lack the target Ag entirely or express it at a level too low for effective IT-mediated killing.

Cloning and expression of actinoporins has been important for the biotechnological applications of these proteins. It has become possible to apply the biotin-avidin conjugation method to produce ITs containing sea anemone cytolysins (figure 2A). Homogeneous and well characterized preparations were obtained when mutants of EqtII were biotinylated with biotin maleimide, a reagent able to react specifically with the single cysteine introduced in the mutants. On the other hand, enhancement of the biotinylated EqtII activity was observed in the presence of the complete targeting complex (primary antibody and a secondary avidin-conjugated antibody. However, the non-specific toxicity of actinoporin based IT remains a difficulty. The main problem of most of the ITs containing membrane active proteins is the poor cellular specificity associated with the capacity of the toxin to bind to almost any cell membrane.

An approach designed to overcome the non-specific toxicity is the construction of inactive PFTs with built-in biological "triggers" that will active the toxin following a biological stimulus. Researches have took advantage of the fact that many tumour tissues and tumour cell lines overexpress certain proteases, particularly cathepsin B[26], matrix metalloproteinases (MMPs)[27] and urokinases[28], in the evaluation of tumour protease-activated engineered PFTs.

Promising results using this approach have already been obtained with engineered *Bacillus anthracis* lethal toxin containing either MMPs or urokinase plasminogen activator cleavage sites.

Such approaches need a comprehensive knowledge of the structure-function relationship of the toxin to be used. The three-dimensional structures of EqtII[31,32] and sticholysin II[33] have been solved by NMR and X-Ray crystallography. They are single-domain proteins consisting of a two five-stranded β-sandwich fold with a hydrophobic core and a pair of α-helices, each of which is associated with a side of a β-sheet (figure 3A). These proteins are relatively rigid in solution except for the N-terminal region that can be displaced without disrupting the general fold of the molecule. In the multi-step pore formation process the toxin binds the lipid bilayer with the aromatic amino acid cluster located on a broad loop at the bottom of the molecule and on the C-terminal α-helix[34-36] and also by a phosphorylcholine binding site[33]. In the next step the N-terminal segment translocates to the lipid-water interface where it lies flat on the membrane[33,36,37]. Finally four toxin monomers oligomerize[20,38] and the transmembrane pore is formed by the insertion of four N-terminal α-helices, one from each monomer, and most probably also by membrane lipids in a so-called toroidal pore arrangement[39,40](figure 3B).

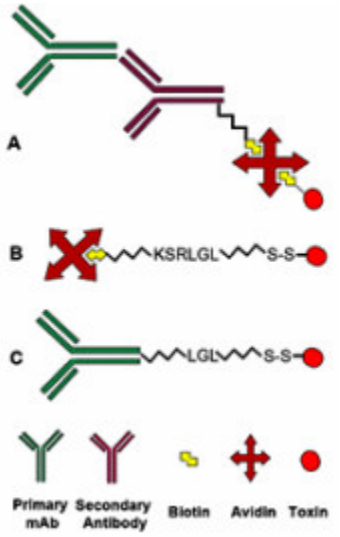

Figure 2. Schematic structure of different constructs using recombinant actinoporins. (A) Avidin-biotin based ITs. Single cysteine mutants of EqtII were targeted to the surface of either *Giardia*[25] or human malignant melanoma cells[12] using avidin-biotin based ITs. In such constructions unwanted cells are recognized by a targeting block which is composed of a primary mAb specific for an Ag expressed on the surface of the cell, a biotinylated secondary antibody and avidin and after that a biotinylated mutant is applied. Avidin connects the targeting block to the biotinylated toxin and brings it closer to the surface of the cell, where it can act on the lipid membrane. (B) Tumour proteinase-activated pore-forming toxin. The pore forming capacity of EqtII was reversibly blocked by conjugation of avidin to its N-terminal extreme. The mutant EqtII I18C was bound through a disulfide bond to a peptide containing cleavage sites for the tumour-associated proteinases MMPs (LGL) and cathepsin B (KSRL) and biotin at the C-terminal extreme for binding avidin. (C) Tumour proteinase-activated immunotoxin. The binding capacity of an actinoporin mutant would be reversibly blocked by conjugation of a mAb to binding region using peptide containing a cleavage site for tumour-associated MMPs (LGL).a

Actinoporins exhibit two very convenient characteristics for designing tumour proteinase-activated toxin conjugates. The extreme stability towards proteolytic degradation makes them good candidates for this application. The lack of cysteine residues in their primary structure is also advantageous since this reactive amino acid can be introduced at a desired position by site-directed mutagenesis in order to make possible a side-directed conjugation.

In the first trial a tumour proteinase-activated actinoporin was constructed[41]. A normally active mutant of EqtII (I18C) containing one single cysteine in the amphiphilic N-terminal α-helix was linked to the protein avidin via a biotinylated peptide including cathepsin and MMP cleavage sites (figure 2B). The conjugate obtained was able to bind all the studied cell types (erythrocytes, fibrosarcoma and breast carcinoma cells).

However, contrary to the null effect observed on erythrocytes (devoid of cathepsins and MMPs enzymes), the conjugate displayed cytotoxic activity on the tumour cell lines where a good correlation between the cell's sensitivity and cathepsin B activity was also found. A strong indication that some MMP activity was also involved in the proteinase-activated actinoporin cytotoxicity was the observation that the human breast adenocarcinoma cell fraction killed by the conjugate treated with the specific MMP inhibitor GM6001 was one-fifth com-

pared to the one without inhibitor.

These results demonstrate the possibility of tumour cell killing by an actinoporin based conjugate specifically activated by tumour proteinases. Other possible applications for such constructs would be the delivery of low molecular weight agents encapsulated in liposomes carrying inactive pores that can be activated at the tumour site by the action of specific proteinases.

However, the tumour proteinase-activated PFT obtained in this work retained its ability to bind to any type of cell. Thus, for construction of an actinoporin-based tumour proteinase-activated IT lacking non-specific activity, the mAb should be coupled through the cleavage peptide to the toxin binding region, thereby preventing the unactivated toxin from binding to the cells. Thus, the conjugates by themselves should be non-cytotoxic to normal cells, by this means improving their therapeutic index. Design of such a therapeutic agent would take advantage of two separate properties exhibited by cancer cells (expression of tumour-associated Ags and overexpression of proteinases) and would allow a single hybrid molecule to recognize the tumour cell through its ligand moiety and be specifically activated at the tumour site by the tumour-associated proteinases. A schematic representation of a tumour proteinase-activated IT is shown in figure 2C.

In this direction we have obtained a mutant of StI (StI W111C) with a cysteine residue in the cytolysin binding region in order to construct tumour proteinase-activated ITs as those described previously. The construction of such ITs is in development.

Table I
Biological activity of ITs constructed with actinoporins

Toxin	Targeting molecule	Receptor	Cells	Cytotoxic Activity, C_{50}
Hemolytic fraction from *Stichodactyla helianthus*	IOR-T6 mAb	IOR-T6 (Antigen expressed on immature T-lymphocites)	CEM (Human T-acute lymphoblastic leukemia cell line)	2×10^{-9} M*[a]
	CB-CEA-1 mAb	CEA (Carcinoembriogenic antigen)	MDA-MB-134 (Human brest carcinoma cell line)	8×10^{-9} M*[b]
	IOR egf/r3	Human epidermal growth factor receptor	H125 (Human Lung adenocarcinoma cell line)	1×10^{-9} M*[c]
StI	Ior C5 mAb	Ior C2 (Antigen expressed on colorectal and ovarian cancer cells)	SW 948 (Human colorectar cancer)	25×10^{-8} M[d]
EqtII	Diferric-Transferrin	Transferrin receptor	Raji (Human Burkitt lymphoma cell line)	2×10^{-9} M**[e]
				1×10^{-10} M***
IT constructed with recombinant actinoporins				
EqtII A179C	Anti-*Giardia* mAb	Antigen on the surface of *Giardia* cells	*Giardia duodenalis*	1.25×10^{-9} M*[f]
EqtII K20C	OKT9 mAb	Transferrin receptor	A 375 (Human malignant melanoma cell line)	4×10^{-8} M[g]
EqtII R126C				1.7×10^{-8} M
EqtII A179C				3.5×10^{-9} M

[a] Reference [7]; [b] Reference [8]; [c] Reference [9]; [d] Reference [10]; [e] Reference [11]; [f] Reference [25]; [g] Reference [12].
*Value estimated from the original dose-dependence graphic; **Acute toxicity test; ***Long term toxicity test

3 Potential for future development

Although the mechanism of inducing cell death mediated by actinoporin based IT could be the imbalance provoked by the formation of pores in membranes, a promising alternative is the use of such entities as components of chemotherapeutic cocktails. By facilitating drugs to enter into the cytosol, actinoporins might be used to increase the efficacy of conventional anti-cancer agents. In fact it was recently shown that Bc2, an actinoporin isolated from *Bunodosoma caissarum*, and EqtII significantly potentiated the cytotoxicity induced by low dose concentrations of the chemotherapeutic agents cytosine arabinoside, doxorubicin, and vincristine[42]. The induction by these drugs of a more effective cytotoxicity when combined with actinoporins could allow the reduction of their therapeutic doses and consequently their undesirable side effects.

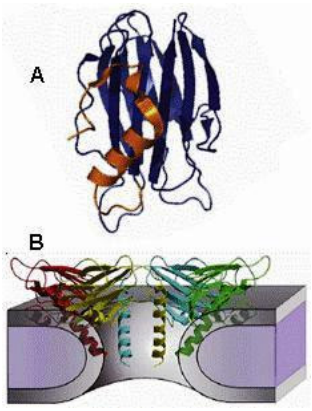

Figure 3. Overall Structure of StII. (A) Stereo view ribbon diagram of StII. The N-terminal region is shown in brown. (B) Putative model for the functional pore of StII, in which the N-terminal region of the protomers would adopt a helical conformation. Lipid headgroup regions are shown as gray layers. The walls of the pore would be formed both by lipid molecules and four N-terminal helices. Taken from reference [33].

Toxins that have been used to make classical ITs consist of several domains and typically one of them is a

recognition domain. This has allowed the lack of inherent specificity of these ITs to be overcome by removing the binding domain. This approach did not seem possible for actinoporin based ITs, since actinoporins are 20 kDa compact structures in which at least two regions, the N-terminal and the Trp-rich binding regions, become embedded in the lipid membrane.

Recent studies have demonstrated that the N-terminal region of actinoporins forms a stable helix long enough to span a lipid bilayer when in a membrane environment although the isolated peptides are only weakly active[43,44]. Thus, while the N-terminal region has the structural features required for the mechanism of pore formation, the lower activity of the isolated N-terminal region shows that the bulk of the protein is essential for efficient pore formation by facilitating initial membrane binding, interaction with SM, or stabilization of the oligomeric pore.

These results suggest that it would be possible to recover, at least in part, the whole toxin pore-forming activity if the N-terminal region were linked to a binding ligand as a growth factor or an Fv fragment. In fact the first artificial native-like pore-forming protein showing selectivity toward malignant cells has been conceived and designed based on the structure of the colicin family of bacteriocins[45]. In the same direction a recombinant IT containing the N-terminal fragment of human perforin, a pore-forming molecule that mediates human cytotoxic T-cell killing, has been engineered and evaluated *in vitro* with promising results[46].

The relatively small fusion protein, if it recovers the cytotoxic activity, might be specifically toxic to a cell population. Alternatively, the binding ligand could concentrate the N-terminal peptide on the surface of the target cell and a tumour proteinase N-terminal activation mechanism, similar to the one previously described above, could be introduced. In such a construction an increase of the specific N-terminal peptide cytotoxic activity toward the target cell would be expected.

These last approaches have some additional advantages, as they would allow production of recombinant actinoporin based ITs, which is not possible with the whole protein since the binding region, present in the *middle* of the primary structure, must be blocked to reduce the non-specific activity. In addition, recombinant ITs would be homogeneous and relatively inexpensive to produce. The reduction of the possible immunogenicity associated with the full length toxin would also be desirable. However, at present, little is known about the immunogenic properties of actinoporins.

4 Conclusions

Although the first *in vitro* evaluations of ITs containing sea anemone cytolysins are encouraging, advances in their application have been limited by a lack of knowledge about their 3D structures and structure-function relationships. The results obtained using anthrax lethal toxin targeted to either uPA or MMP-expressing tumour cells suggest that proteinase-activated anti-tumour toxins have considerable potential for use in cancer therapy. Tumour cell killing by an actinoporin-antibody conjugate that is specifically activated by tumour proteinases would include two levels of specificity: antibody targeting of tumour Ag and activation of the actinoporin by tumour proteases.

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