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Structure Based Design of Cholera Toxin Antagonists

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1. Introduction

Cholera is an acute enteric infection, with huge pandemic potential, caused by ingestion of food or water contaminated with the bacterium *Vibrio cholerae*, the gram negative bacteria (Sack, Sack et al. 2004). The etiologic agent responsible for cholera was identified in 1883 when Robert Koch demonstrated that comma(-shaped) bacteria, later designated as *V. cholerae*, causes cholera infection (Koch 1884). Since Koch's discovery of cholera virulent factor, different specific strain variants of *V. cholerae* have been identified. For the majority of cholera's disease outbreaks two biotypes of *V. cholerae's*erogroup O1 are responsible: Clasical and "El Tor", as well serogroup O139 that was responsible for a large epidemic in Bangladesh and India (Ramamurthy, Garg et al. 1993). Non-O1 and non-O139 *V. cholerae* can cause mild diarrhoea but do not generate epidemics (Ramamurthy, Bag et al. 1993). Cholera transmission is closely related to inadequate environmental conditions that can be find in suburban slums where the basic infrastructure is not available, as well as in camps for internally displaced people or refugees, where minimum requirements of clean water and sanitation are not met. A typical example of such non-promising situation has induced an outbreak of Cholera after earthquake in Haiti in January 2010 (Andrews and Basu 2011). The short incubation period of two hours to five days, enhances the potentially explosive pattern of outbreaks. Intensive efforts for the identification of the basis of Cholera disease at a molecular level were done by different research groups during the 1960s, until Finkelstein and co-workers recognized a protein toxin as a major virulent factor that causes the massive fluid release in Cholera infection (Finkelstein, Atthasampunna et al. 1966). The efforts to solve a complete structure of Cholera toxin by X-ray diffraction analysis were concluded during the 1990s (Spangler 1992; Zhang, Scott et al. 1995). Up to date, 27 X-ray structures related to Cholera toxin (CT) are deposited in the Protein Databank. The structure and function of CT at the molecular level will be the subject of our review. In this work we will also show some examples of structure based design of various types of CT inhibitors; we will introduce catechin-like compounds as inhibitors of the enzymatic A unit of CT; mimics of oGM1 as inhibitors of the non-toxic pentamer of B subunits of CT (CTB); as well as multivalent inhibitors that very effectively prevent adhesion of CTB to GM1 receptors at the surface of epithelial cells. At the end, we will also describe a new strategy for developing inhibitors via targeting binding site for blood group antigens in Cholera Toxin.

2. Cholera toxin – structure and mode of action

Cholera toxin (CT) belongs to the family of AB_5 bacterial toxins, which includes CT itself and the *Escherichia coli* heat-labile toxins (LTs) LT-I and LT-II, among others. This family of bacterial toxins is named after the characteristic architecture comprising a single catalytically active component, A, and a non-toxic pentamer of B subunits (B_5) (Fig. 1a). The structure and function of AB_5 toxins have been reviewed in detail on several occasions (Bernardi, Podlipnik et al. 2006; Hol, Fan et al. 2004; Hayes, Turnbull 2011).

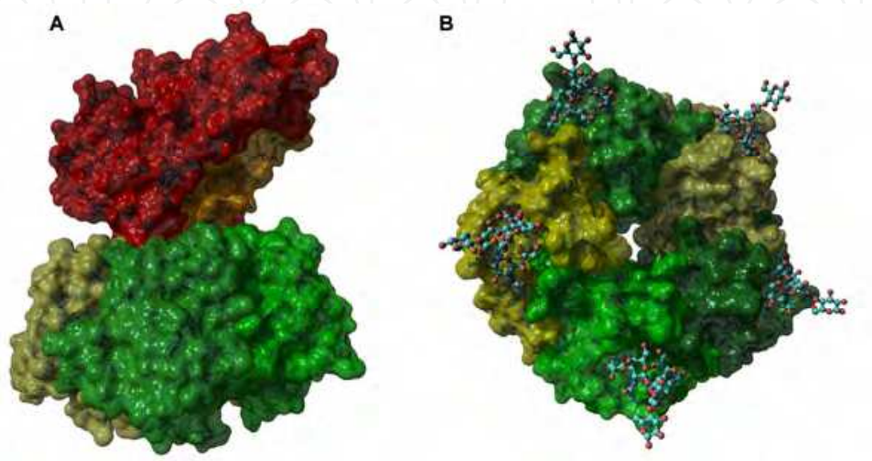


Fig. 1. (A) Holotoxin CT and (B) pentamer B complexed with oGM1.

The A subunit of CT is composed of two distinct parts A_1 and A_2. The A_1 component is responsible for the toxic enzyme activity, while the A_2 component serves as non-covalent linker of subunit A to subunit B. Each of five CT's B-subunits is composed from two α helices and two three-stranded β sheets, that form together a doughnut-shaped structure, which has a central pore into which the C-terminal of A_2 subunit extends. The B pentamer is responsible for binding CT to ganglioside GM1 on the external membrane of intestinal epithelial cells. This binding is recognized as a key event for initiation of the threatening action of CT. The interaction of the oligosaccharidic head groups of ganglioside GM1 (Galβ1-3GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glcβ1-OH,oGM1) with B_5 unit of CT is depicted in Fig. 1b. It is interesting that the binding ability of the B-pentamer to cell surface receptors is retained even in absence of the A-subunit. However, the complete AB_5 holotoxin is needed for actual intoxication.

The B pentamer of CT (CTB) interacts with the soluble, monovalent oligosaccharide portion of GM1 (oGM1) with strong affinity, the binding process is weakly cooperative. The close view of the interaction based on 1.25 Å resolution structure of oGM1:CTB complex (Merritt, Sarfaty et al. 1994) is shown in Fig 2b. We may observe that branched oGM1 (Fig 3) is attached to CTB binding site with two fingers: the first one is a sialic acid "thumb" and the second one a GalB(1->3)GalNAc "forefinger" (two-fingered grip). Most of the contacts are given by the "finger" tips: in terms of buried protein surface, the terminal Gal and Neu5Ac residues contribute 39% and 43%, the rest and minor part of protein surface is buried with GalNAc. The most recent value of the dissociation constant for interaction between one oGM1 and one CTB binding site has been evaluated by Isothermal Titration Calorimetry (ITC) and it was found to be 43 nM (Turnbull, Precious et al. 2004), this is one of the

strongest known protein-carbohydrate interaction. It has been also observed that all of the mono- or disaccharide fragments of oGM1 bind to CTB much more weakly; for example, the terminal galactose residue displays K_d=15 mM, which is in the case of the Gal-GalNAc forefinger improved by only a factor of two.

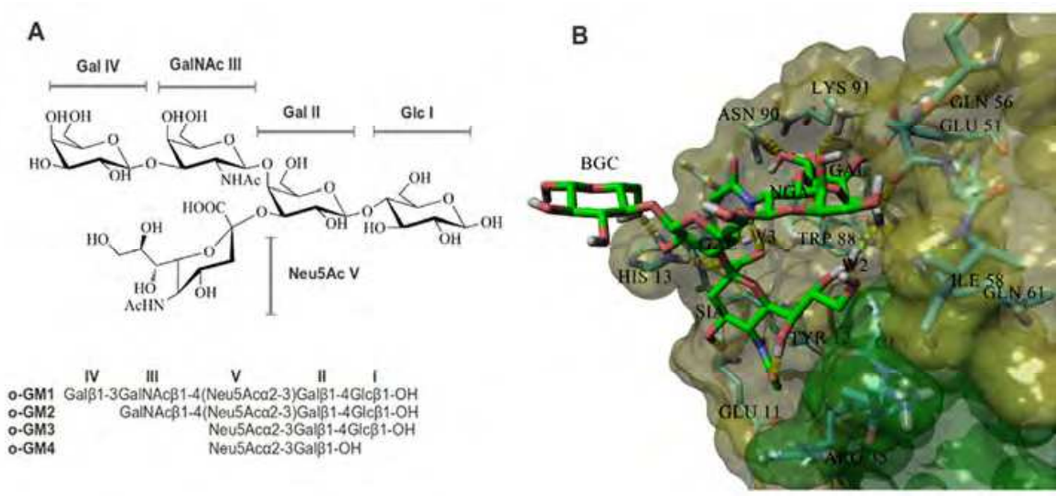


Fig. 2. (A) *Structure of ganglioside head groups. (B)* Close view of CTB:oGM1 interaction.

The other important binding determinant, Neu5Ac, binds even more weakly to the protein (K_d ≈ 200 mM). The analysis of results obtained by dissecting of the oGM1:CTB interaction by ITC has shown that high affinity and selectivity of oGM1:CTB interaction originates mainly from the conformational pre-organisation of the branched GM1 pentasaccharide rather than through the effect of cooperativity of terminal moieties galactose and sialic acid. The terminal galactose residue in the "forefinger" binds to the CTB binding site very specifically. The pyranose ring of this galactose is stacked on top of Trp88 (CH/pi interaction) and forms an extensive hydrogen bond network involving Asn90, Lys91, Glu51 and Gln61 residues from CTB_5. The terminal galactose is docked in a deep cavity that is shielded from the solvent, on the other hand the rest of the toxin's binding site is shallow and solvent exposed. The sialic acid (Neu5Ac) represents the second important moiety of GM1 required for recognition of Cholera toxin. The sugar ring of sialic acid makes hydrophobic interaction with Tyr12, whereas hydroxyl, and N-acetyl substituents form hydrogen bonds with CTB residues, while carboxyl group forms water-mediated hydrogen bond with Trp88.

The action of CT is initiated by binding of CT via the B_5 pentamer to GM1s receptors that are part of the external membrane of intestinal epithelial cells. When CT binds to the cell, the whole toxin is transferred into the cell via receptor-mediated endocytosis. The toxin is then transported to the endoplasmic reticulum (ER), where A subunit dissociates from the rest of the protein assembly. Subunit A is then split to subunits A_1 and A_2 by peptide-disulfide isomerase in the ER. Subunit A_1 is then translocated to the cytosol of the host cell, where it catalyses the covalent transfer of an ADP-ribose moiety from NAD^+ to Arg201 of the signalling protein $G_{s\alpha}$ (a component of an adenylate cyclase system). Adenylate cyclase system is normally activated by a regulatory protein G_s and GTP; however the activation is normally brief, because another regulatory protein (G_i) hydrolyses GTP. The normal situation is described in Fig 3a. Cholera toxin catalyses transfer of ADP to adenyl cyclase

cycle. Ribosylated form of G_s stabilizes the GTP bound form of protein, which stays continually activated (Cassel and Pfeuffer 1978). This situation is shown in Fig 3b.

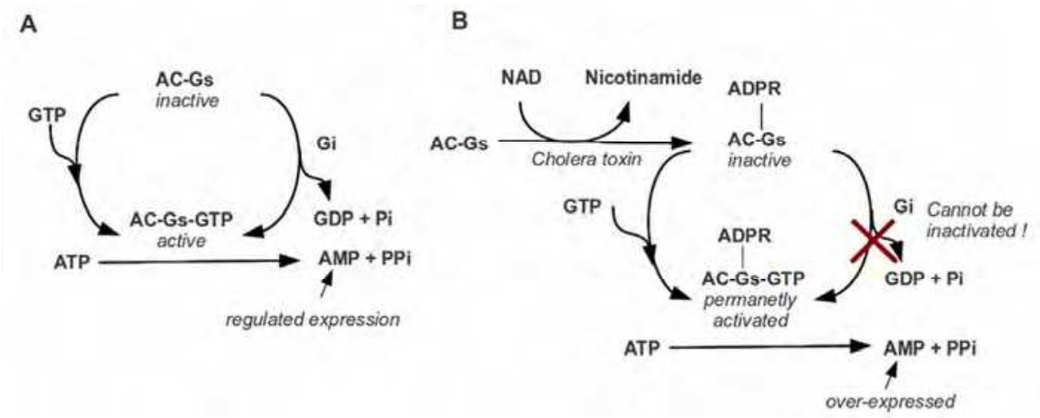


Fig. 3. Action of enzymatic unit of Cholera Toxin. (A) Normal action. (B) Permanent activation of Adenylate cyclase system.

This modification of the adenylate cyclase system results in an elevated level of AMP which causes the activation of the sodium pumps in the lumen of the cells through an AMP-dependent kinase pathway, forcing the Na^+ ions out. The electrochemical imbalance is then compensated by driving Cl^- and H_2O out of the cells. The process of Cholera Toxin action is followed by enormous loss of water from the epithelial cells into the intestinal lumen, causing water diarrhoea characteristic for cholera.

It has been shown that A_1 by itself has relatively low enzymatic activity in vitro. The interaction of A_1 with ADP-ribosylation factor (ARF) protein from human host increases the enzymatic activity of A_1. Numerous studies *in vitro* and *in vivo* have indicated the importance of tight interaction between A_1 fragment and ARF. Recent structural investigations of a CTA_1 :ARF_6-GTP complex pointed out that binding of ARF_6-GTP causes dramatic changes in the CTA_1 loop regions that open the binding site for NAD^+ (Hol, O'Neal et al. 2005).

Taking into account structural information and given mechanism of action of CT, three different strategies are possible to design a prophylactic cure against Cholera:

- Inhibition of the action of the catalytically active unit A of CT.
- Design of small molecules that act as a decoys for the toxin's GM1 binding site and thus prevent adhesion of CT to cell membranes of epithelial cells.
- Prevention of assembly of AB_5 complex that takes place in the cytosol.

In further writing, the first two strategies of the development the Cholera toxin inhibitors will be reviewed, with special attention to experience based on our recent work in this field of medicinal chemistry.

3. Natural products as Cholera toxin inhibitors

During the history, traditional healers have prepared medicaments for the treatment (prevention) of Cholera infections from various medicinal plants. The most common ways to administer such natural remedies are infusions or decoctions that are usually compositions

of numerous natural products. The active substances from medicinal plants can treat Cholera via different pharmacological mechanisms; from the direct antimicrobial against *V. cholerae*, prevention of adhesion of CT to the GM1 receptors at surface of epithelial cells, to direct inhibition of ADP-ribosylation of active unit of CT. The improved understanding of the CT toxicity at the molecular level and the further set up of modern biological assays, has allowed in recent years the identification of different classes of bioactive natural products. An important class of such products are polyphenols from green and black tea, green apples, hop bract and the Chinese rhubarb rhizome. Garlic extract is another example of traditional cure against diarrhoea diseases such as cholera. Recent researches have recognized a galactan polysaccharide as a major anti-choleric component of garlic (Politi, Alvaro-Blanco et al. 2006). Some interesting natural inhibitors of CT are shown in Fig 4.

Toda *et al*. (1992) reported that polyphenol catechins (EGC, 3, ECG, 4, and EGCG, 5) isolated from green tea have protective function against infection with *V. cholerae* O1. EGCG and ECG also protect against hemolysin (another toxin from *V. cholerae* that causes red blood cell rupture) in a dose dependent manner–the more green tea catechins, the better the protection. Animal studies also showed that these catechins reduced the fluid accumulation (the primary cause of cholera fatality) from CT (Toda, Okubo et al. 1992).

Toda *et al.* (1991) have also suggested that extracts of black tea have anti-bactericidal function against *V. cholerae* O1. The major active components of black tea that could be responsible for protective activity against *V. cholerae* O1 are theaflavin-3,3'-digallate, 9 and thearubigin, 10.

Saito *et al.* (2002) have shown the anti-choleric activity of natural polyphenols extracted from immature apples. They described that the inhibitory effect of apple polyphenols extract (APE) on CT-catalyzed ADP-ribosylation of agmantine is dose-dependent and it is due to the inhibition of the enzymatic activity of the A subunit of CT. The concentration of APE at which 50% of the enzymatic activity of CT (15 µg/ml) is inhibited was approximately 8.7 µg/ml. Bioassay oriented fractionation of APE indicated that the highly polymerized catechins, also named procyanidine polymers, are the major inhibitory components of this apple extract. Other constituents like the non-catechin-type polyphenols (chlorogenic acid, phloridzin, phloretin, caffeic acid, and p-coumaric acid) and the monomeric catechins (catechin and epicatechin) have shown week inhibitory activity. The results indicate that APE disturbs the biological activity of CT *in vivo*, also but not only via inhibition of the enzymatic activity of A-subunit. An additional explanation for the *in vivo* reduction of secretory activity of APE can also be the protection of the intestine's mucosa with polymerized catechins. Procyanidins B1, 6, C2, 7 and tetracatechin, 8 representative structures from Saito study are shown in Fig. 4.

Hor *et al.* (1995) also reported *in vivo* CTA inhibitory activity of proantho-cyanidines extracted from *Guazuma ulimfolia,* a medicinal plant used in Mexico for traditional treatment of diarrhoea.

Oi *et al.* (2002) studied the bioactivity of rhubarb galloyl tannin (RG-tannin), a compound isolated from *Rhei rhizome,* against CT activities including ADP-ribosylation and fluid accumulation. This kind of heterologuos polyphenol gallate inhibits fluid accumulation in mouse and rabbit ileal loops that is induced by CT action, as well as catalytic activity of

CTA. It was also observed that RG-tannin, had no effect on the binding of CTB to the ganglioside GM1 or an endogenous ADP-ribosylation of membrane proteins. The authors prepared and tested a small library of synthetic gallates (sugar moieties esterified with galloyl groups) against CTA's ADP-rybosylation activity a small library of synthetic gallates. Some of compounds (12 for example) from their library exhibited strong inhibitory activity of CTAs ADP-ribosylation.

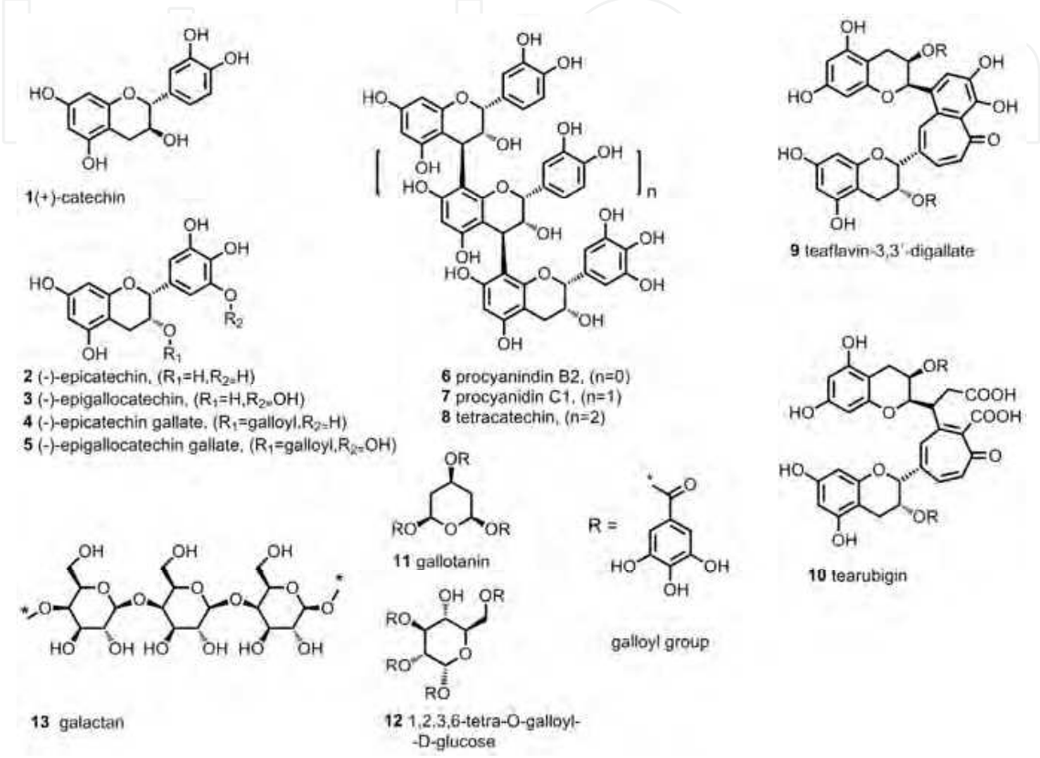


Fig. 4. Structures of natural products that could serve as CT inhibitors.

Politi *et al.* (2006) reported binding activity between a high molecular weight polysaccharide, galactan from garlic water extract and the B subunit of CT (CTB). The interaction was confirmed with Saturation Transfer Difference (STD) experiment, one of the NMR methods used to measure interaction between ligands and target receptor, and with fluorometric binding assays. This study indicates that one galactan polymer could bind with large number of CTB protein monomers. The ability of galactan to form high molecular weight aggregates with CTB and thus prevent adhesion to cell-surface could be the main reason for its inhibitory activity. A fragment of galactan 13 is shown in Fig. 4.

Podlipnik (2009) has collected polyphenol structures from different sources and described a structure-based model of inhibition of ADP-ribosyltransferase activity of Cholera toxin by polyphenol's. Compounds 1-12 (Fig 4) are members of the polyphenol´s library used for virtual screening against CTA_1 . For docking purposes a model based on a crystallographic structure of CTA_1:ARF_6-GTP complex (Hol, O'Neal et al. 2005) was prepared. From docking (Glide XP) results it is evident that mono catechines can penetrate deeply into the binding site of CTA (Fig. 5a). The inhibitory activity of polyphenols generally increases with their complexity, measured by number of hydroxyl groups attached to the scaffold. On the other

hand, oligocatechins can not penetrate into the binding site in the whole extension, such compounds could additionally form numerous non-specific contacts with the protein surface, and hinder NAD^+ to access the binding site (Fig. 5c). Nice fits to CTA binding site have been also observed for theaflavin-3,3 digallate (Fig. 5d), 9 from black tea and Oi's synthetic gallate (Fig. 5b), 12, the results of molecular docking (Glide XP) indicate that these two compounds could act as strong inhibitors of CT´s ADP-ribosylation activity .

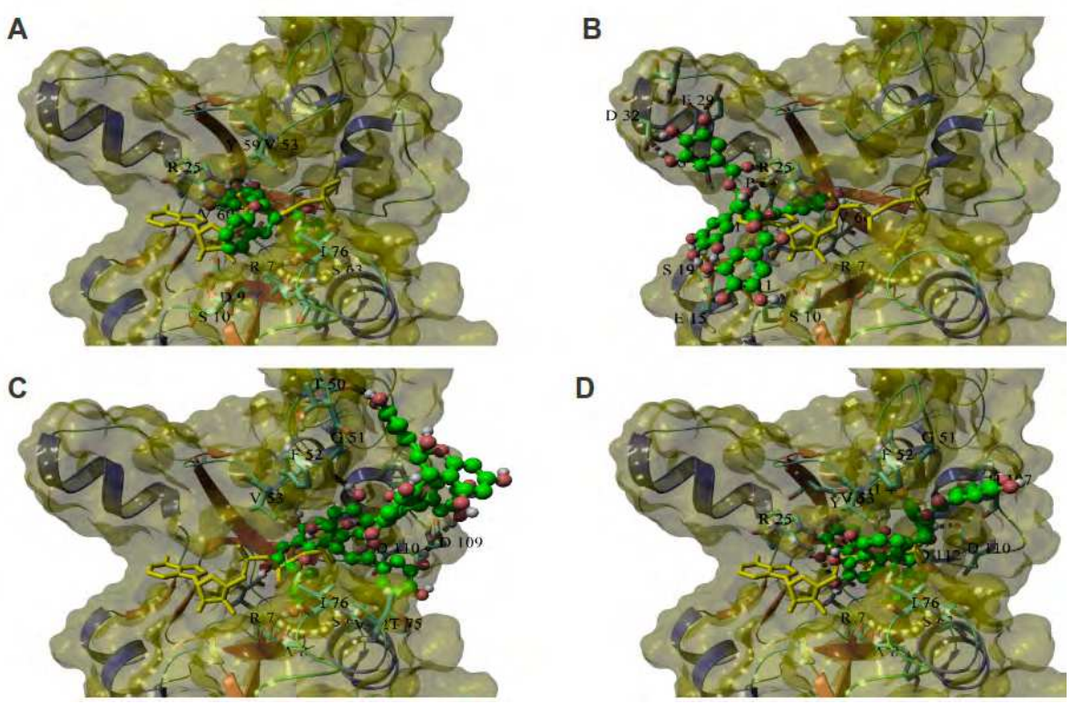


Fig. 5. Docking poses (GlideXP) for selected polyphenols. (A) epigallocatechin gallate; (B) 1,2,3,6-tetra-O-galloyl-D-glucose; (C) tetrameric catechin; (D) theaflavin-3,3-digalate. Yellow coloured NAD+ is appeared in each CTA:ligand complex as a reference.

Many other plants have been used for centuries around the world in traditional medicine as natural remedies for cholera and other diarrhoeal infections. Most of the current pharmacological studies are oriented to test antibacterial activity of some of these medicinal species from plant extracts against *V. Cholerae*. On the other hand, direct investigations of natural products as potential CT inhibitors are very rare, this field of research is still open and some additional founding of direct biological action of natural compounds to cholera toxin and other AB_5 toxins are more than welcome.

4. Rational design of GM1 mimics as Cholera toxin inhibitors

The synthesis of ganglioside GM1 itself is very complex (Sugimoto, Numata et al. 1986), therefore one of the strategies how to prevent adhesion of CTB to the cell surface involves design and synthesis of functional and structural mimics of oGM1 (Fig 6).

NMR and theoretical studies of the conformational behavior of GM1 and of other ganglioside head groups (e.g. GM2, GM3, and asialo GM1) have shown that the 3,4-

branching at Gal-II residue is the main reason for rigidity of oGM1 structure, so Gal-II residue appears to act as the scaffold that holds together the two terminal Gal-IV and Neu5Ac moieties at the proper orientation for optimal interaction with CT (Bernardi and Raimondi 1995; Bernardi, Arosio et al. 2002). Bernardi *et al.* have used the above structural hypothesis to develop and design ligands (14 for example) using conformationally restricted *cis*-1,2-cyclohexanediol, 15, as a replacement of Gal-II residue in oGM1 (Bernardi, Checchia et al. 1999; Bernardi, Arosio et al. 2001). The experimental results obtained by ELISA assays and fluorescence titration have shown that CT inhibition activities of Bernardi's mimics and oGM1 are more or less in the same range. The major problem in the synthesis of the "first generation" of the Bernardi's mimics is the stereoselective syalilation of *cis*-1,2-cyclohexanediol, this step represents the bottleneck of the synthesis. Therefore, a further simplification of oGM1 structure that has been based on the replacement of the sialic acid residue with simple α-hydroxyl acids, 16-20 was proposed by Bernardi's research group (Bernardi, Carrettoni et al. 2000).

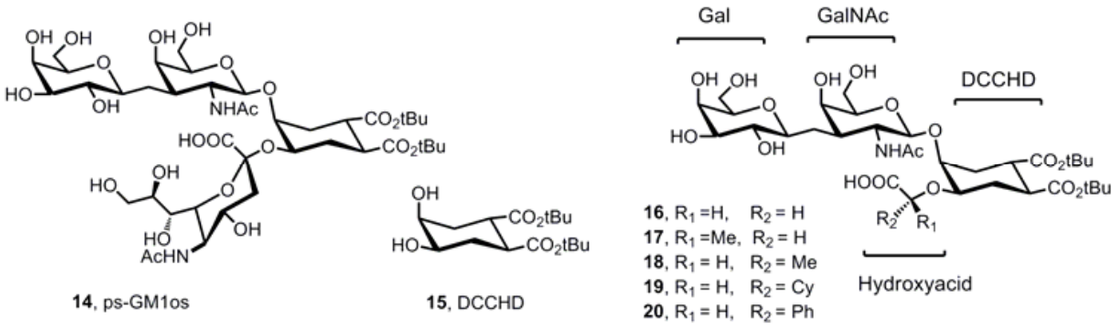


Fig. 6. Structures of Bernardi's GM1 mimics.

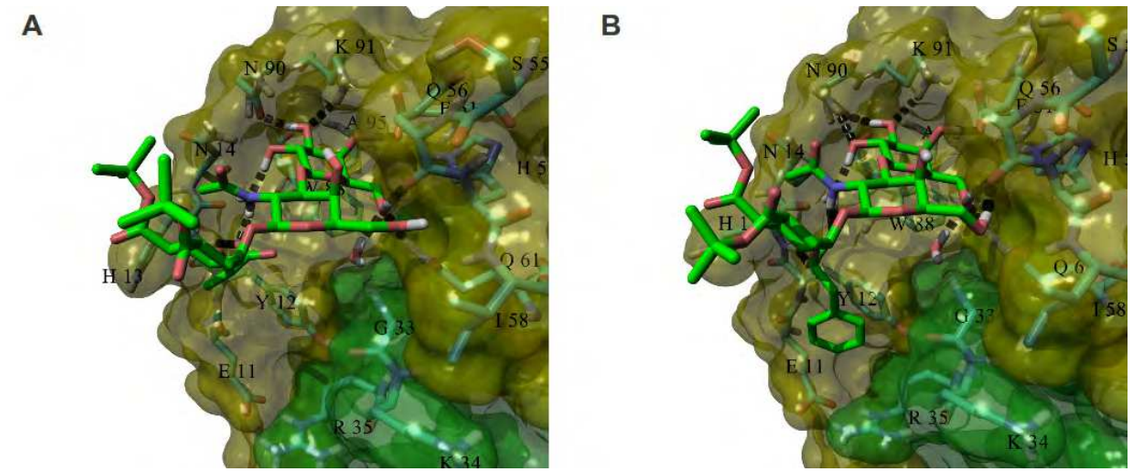


Fig. 7. Poses (Glide XP) of Bernardi's GM1 mimics: (A) 18 and (B) 20 to CTB .

The next widely used approach to design CTB inhibitors is to use the terminal galactose as an anchor to which various pharmacophores can be attached. Minke *et al.* (1999) have used fluorescence titrations and ELISA assays to screen a series of commercially available galactose derivates. The most active compound from this series was meta nitrophenyl α-D-

galactoside (MNPG), 21. Its affinity for CTB (IC_{50} = 720 μM) is two orders of magnitude higher than it is found for galactose. (Minke, Roach et al. 1999) (Fan, Merritt et al. 2001) Further crystallographic studies have shown the displacement of a water molecule that is structurally bound to CTB by the meta nitro group of the MNPG's phenyl ring. The mentioned displacement leads to an increase in the entropy of the system and creates tight hydrogen bond interactions between the nitro group of MNPG and CTB could, which may be the reason for an increased CT inhibitory activity (Fan, Merritt et al. 2001). It has been also observed that m-carboyxphenyl α-D-galactoside (MCPG), 22, binds to the target with a different binding mode. Poses of MCPG and MNPG extracted from crystallographic structures are shown in Figs. 9a and 9b, respectively (Fan, Merritt et al. 2001).

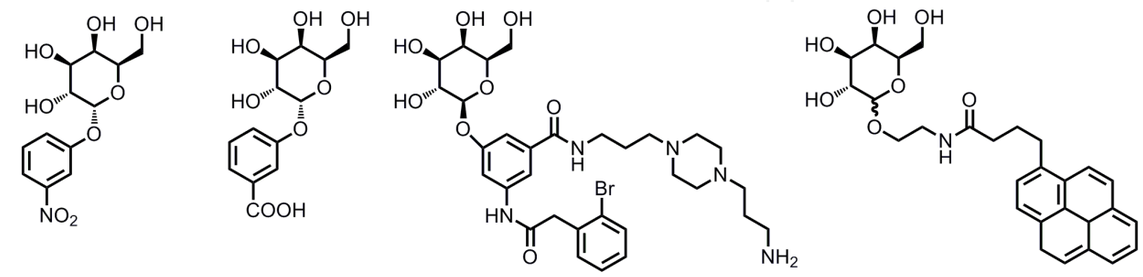


Fig. 8. Some examples from MNPG (MCPG) library.

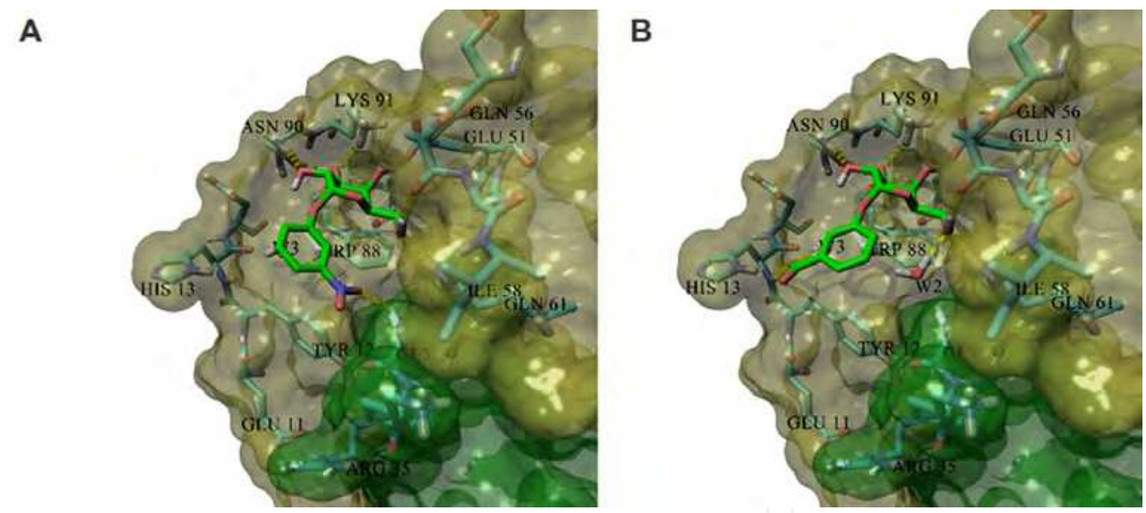


Fig. 9. Poses of (A) MNPG, 21 and (B) MCPG, 22 to CTB. Poses are extracted from crystallographic data.

Fan *et al.* designed a library of CTB antagonists where rigid hydrophobic rings were linked with different short and flexible aliphatic linkers to the meta position of phenyl ring of α-D-galactoside (Fan, Pickens et al. 2002). Some compounds from the mentioned libraries are shown in Fig. 9. This modification of MNPG allows to explore different regions of CTB binding site. Minke *et al.* (1999) explored a hydrophobic pocket in the bottom of the LT-II binding with series of galactosides that have large hydrophobic moieties, and found that compound 24 had the lowest IC_{50}=40 μM, which is more than three orders of magnitude

lower from the IC_{50} of galactose . Docking (Glide XP) pose of 24, an α galactoside with large rigid hydrophobic moiety, to CT is shown in Fig. 12a.

Pieters *et al.* (2001; 2002) synthesized monovalent lactose-derived ligands for Cholera toxin, an example of such ligand is 25, where a thiourea moiety served as a spacer between lactose and aromatic system. A 72 fold enhancement of binding affinity of the compound 25 (K_d=248 μM) versus lactose (K_d=18 mM) to CTB determined by fluorescence titration was observed. The next step to improve Pieters' ligands was to increase the rigidity of a spacer between the lactose and a aryl group. The fluorescence study of 26 (Kd=23 μM) revealed one order of magnitude enhancement in the affinity of 25 for the CTB. Two examples from the library of Pieters' ligands based on lactose scaffold are shown in Fig. 10.

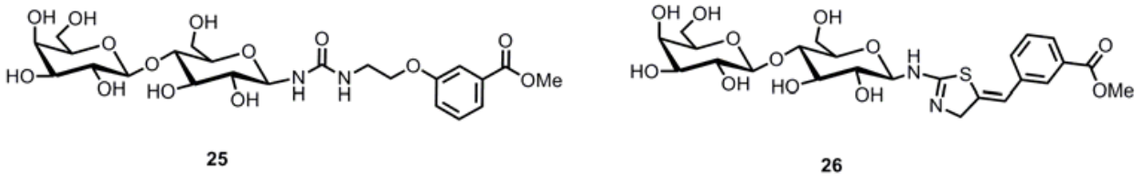


Fig. 10. Pieters' lactose derived ligands.

Mari *et al.* (2004; 2006) designed and synthesized a galactose-derived bi-cyclic scaffold, the rigid framework and possibility of functionalization at appended side-chain made these compounds interesting for further combinatorial development. NMR and conformational search analysis showed, however, that these compounds were more flexible than expected and did not fit the cholera toxin's binding site. An example, 27 from their library is shown in Fig. 11.

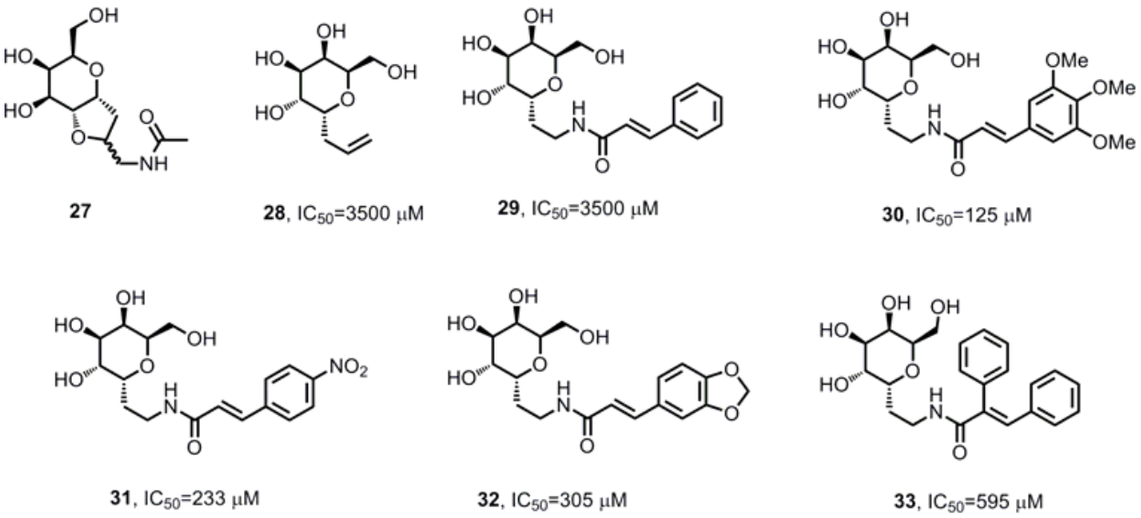


Fig. 11. Cholera toxin inhibitors: bi-cyclic inhibitor, 27 and cinnamic acid galactoconjugates 28-33.

Podlipnik *et al.* (2007) designed a small focused library of functionalized C-galactosides that could serve as non-hydolysable inhibitors of Cholera toxin. The fact that C-galactoside anchors (compound 28 from Fig. 11) can be synthesized in a few steps from galactose, thus avoiding the need for protecting groups, and their metabolical stability are the main reasons

for the selection of the scaffold. The approach that has been adopted to identify CT inhibitors involves the following steps: the development and validation of a docking/scoring protocol based on a set of known pseudo-GM1 ligands; the design of a focused library of C-galactosides; the synthesis and affinity evaluation (by SPR) of selected elements of the library. The authors have tried to design relatively rigid ligands with α configuration on Galactose anomeric center that could fit CTB binding site. Cinnamic acids and their derivatives have been found as an ideal solution for conjugation functionalized C-galactosides (compounds 29-33 from Fig. 11). The best value of IC_{50} (125 μM) has been observed for compound 30. The pose of this compound within CTB binding site (Glide XP docking) is shown in Fig. 12b.

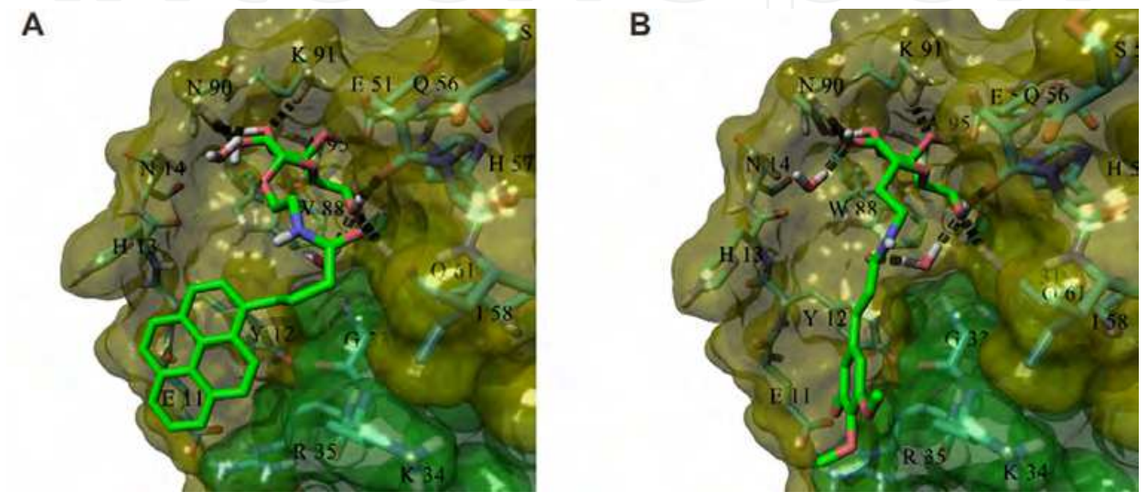


Fig. 12. Docking (Glide XP) poses of (A) Verlinde's ligand 24 and ligand 30 from cinnamic acid galactoconjugates library to CTB. Comparison of two galactoconjugates with α configuration on anomeric centre.

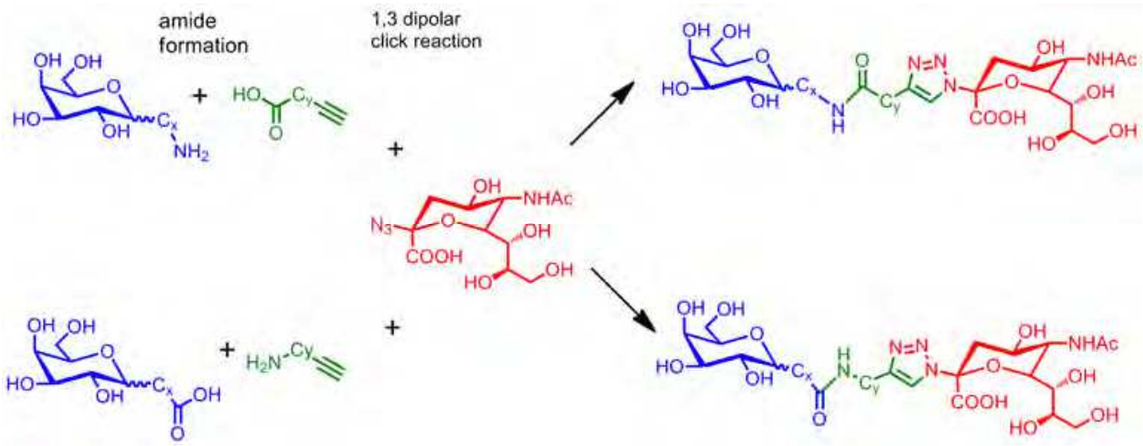
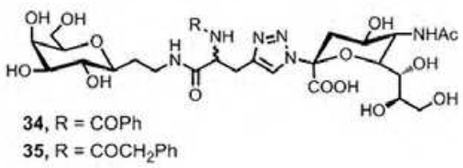


Fig. 13. General scheme for design of non-hydrolysabile Cheshev's bidentate CT inhibitors.

Cheshev *et al.* (2010) have used click chemistry to design a library of non-hydrolyzable bidentate CTB ligand, where two binding determinants, galactose and sialic acid, are connected to one other as they are in oGM1. All compounds from their library were

synthesized from readily available precursors using high performance reactions, including click chemistry protocols, and avoiding glycosidic bonds. The general strategy of Cheshev's design is shown in Fig. 13. The affinity of bidentate ligands to CT measured by weak affinity chromatography could be enhanced up to one or two orders of magnitude relative to the individual pharmacological sugar residues. A further enhancement of CT inhibition could be accessed by conjugation of some of the compounds from the library with polyvalent aglycons. Two examples from Cheshev's library are shown in figure 14. Nice fit computed with Glide XP docking software of *R*-epimeric form of ligand 34 to CTB is shown in Fig. 14.



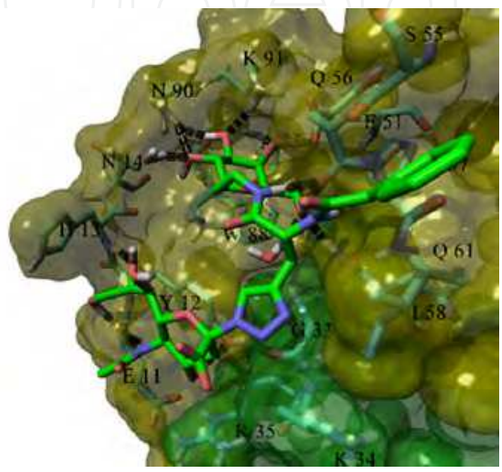


Fig. 14. Two examples from Cheshev´s design and docking (Glide XP) pose of R epimer of compound 35 to CTB.

In this section we shortly described a rational design of CTB antagonists derived from structural simplification of oGM1 – the natural receptor for CT. The presented structures are in the range of very close structurally related mimics of oGM1, such as psGM1, 14 and α-hydroxylacid derivatives, 16-20 with K_d that is close to value found for oGM1 itself, to very simple galactoconjugates MNPG, 21, cinnamic acid galactoconjugates, 29-33 for example. The affinity data for binding of simple galactoconjugates to CTB are still far from ideal, but by conjugation of these compounds with polyvalent aglycons the affinity could be enhanced by several orders of magnitude. The design of multivalent inhibitors will be described in next section of the review.

5. Design of multivalent inhibitors

A very effective way to enhance ligand's binding affinity toward its receptor target is to use the ligand in multivalent presentation. The Cholera toxin B-pentamer is an ideal target for studying multivalency and developing multivalent ligands against its action. Due to its high five-fold symmetry and the fact that it has five identical binding sites for GM1. A first attempt to improve oGM1 affinity using multivalency was reported by Schwarzmann *et al.* (1978), who designed and synthesized divalent oGM1 that has better affinity to CTB than oGM1 by itself. More recently, Schengrund *et al.* (1989) prepared highly active multivalent o-GM1 ligands by linking them to a polymer (poly-L-lisine) or to a dendrimer (octapropyleneimine) that serves as a core.

Fan *et al.* (2002) have designed a multivalent receptor-binding antagonists against CT and LT-II with particular focus on exploiting the 5-fold symmetry of the binding sites on the toxin B pentamer. A conceptual design of such symmetric pentavalent ligand where monovalent "fingers" that block the toxin receptor binding site are attached to symmetric core via modular linker units is shown in Fig. 15. Multivalent inhibitor 36 (Fig. 16) is shown as an example of symmetric pentavalent inhibitor with using MNPGs as monovalent fingers. The affinity of 36 for CTB was investigated using enzyme-linked addhesion assay, from which ED_{50}=0.9 μM was determined. This represents more than 250-fold enhancement of the activity found for MNPG, 21. Crystallographic studies of complex between 36 and CTB brought additional support for a 1:1 association model between the ligand and the toxin, the canonical water is displaced also in this case (Minke, Pickens et al. 2000; Hol, Zhang et al. 2002).

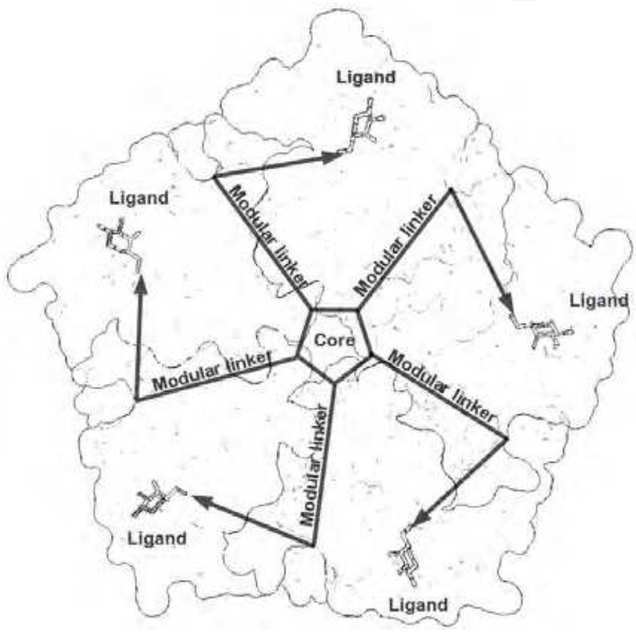


Fig. 15. General scheme of Fan's symmetric multivalent inhibitor design.

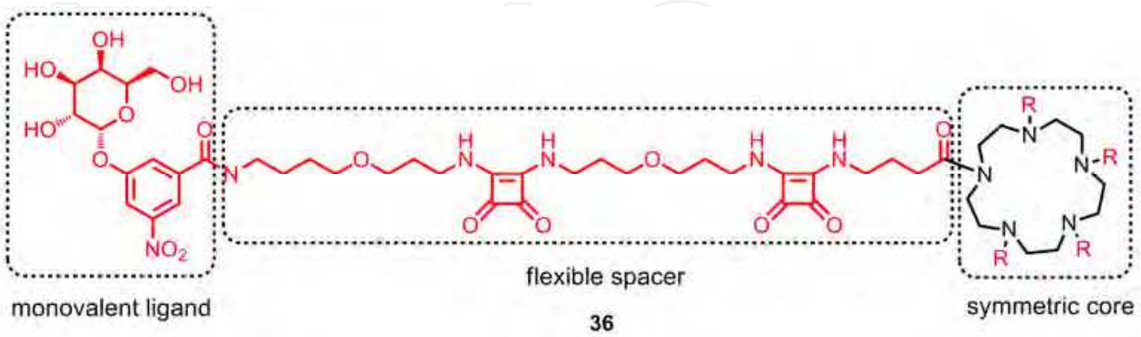


Fig. 16. An example of multivalent inhibitor with pent-fold symmetry designed by Fan.

Another strategy for the design of multivalent inhibitors involves the use of dendrimers. A typical example of such design was shown by Pieters and cooworkers (Pieters, Vrasidas, et al. 2001; Pieters, Vrasidas, et al. 2002), who derived denrimers from 3,5-di-(2-

aminoethoxy)benzoic acid repeating units with 2,4 and 8 end groups to which lactose isothiocyanate units, 25, were attached, providing thioureea-linked glycodendrimers 37 (Fig. 17). Analysis of fluorescence titration data showed that the affinity of these compounds for CT was increased by one order of magnitude relative to the monovalent ligands. It has been also shown that the branching of dendrimer provided only a modest increase in the potency of the ligand. The authors have also reported same indications that ligands are able to bind to multiple toxin molecules, rather than to single B pentamer.

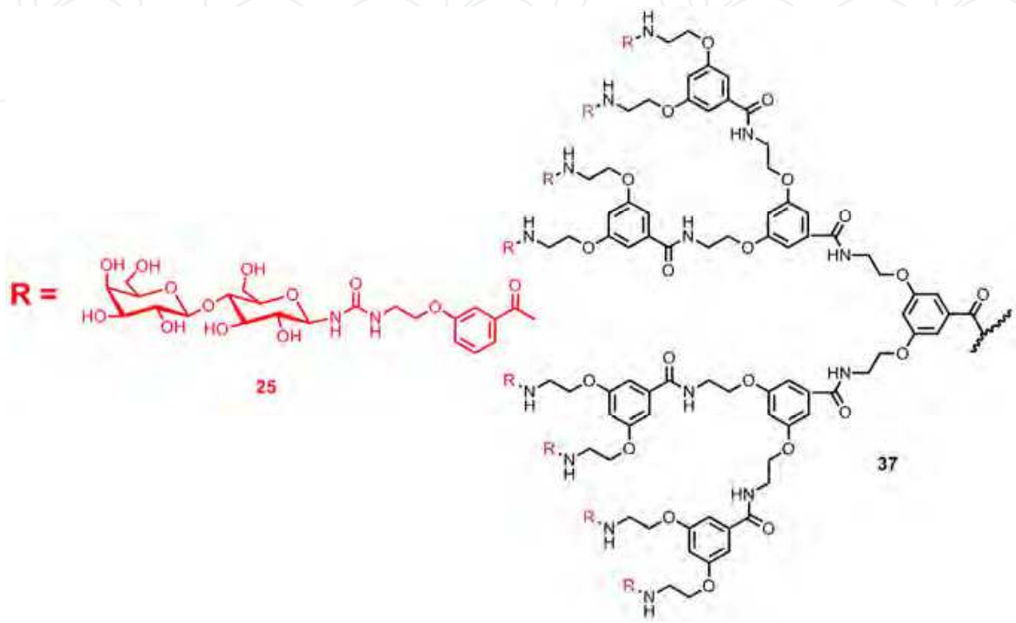


Fig. 17. A scheme of Pieters' multivalent inhibitor based on dendridic structure.

A further improvement of multivalent ligand's affinity for the toxin has been obtained attaching Bernardi's monovalent GM1 mimic 25 to Pieters' dendrimeric core (Arosio, Vrasidas et al. 2004). For further improvement, the polysaccharide scaffold was provided with elongated spacer arms. The analysis of surface plasmon resonance data revealed EC_{50}=0.5 μM for 38.

Another option of using Bernardi's GM1 mimics for multivalent inhibitor's design has been presented by Bernardi, Casnati and cooworkers (Bernardi, Arosio et al. 2005). They prepared a bivalent ligand 39 by hooking two units of GM1 mimic 18 to a functionalized calix-[4]-arene core. The size of affinity enhancement measured by fluorescence titration was found to be 3800-fold (1900-fold per sugar mimic). Recently, the huge affinity enhancement of 39 versus 18 to CT were confirmed in our laboratory with isothermal calorimetry titration (Prislan, et al. 2011).

The best known multivalent inhibitors up-to date have been reported by Pukin *et al.* (2007). In this study, GM1 containing compound was synthetized enzimatically starting from ω-azidoundecyl lactoside, that was then coupled onto Pieters' linker-extended dendrimers by cooper-catalysed azide-alkyne cycloaddition. Dendrimers bearing two, four and eight GM1 sugars were evaluated by ELISA, the IC_{50} values for these compounds were 2 nM, 0.2 nM and 50 pM, respectively.

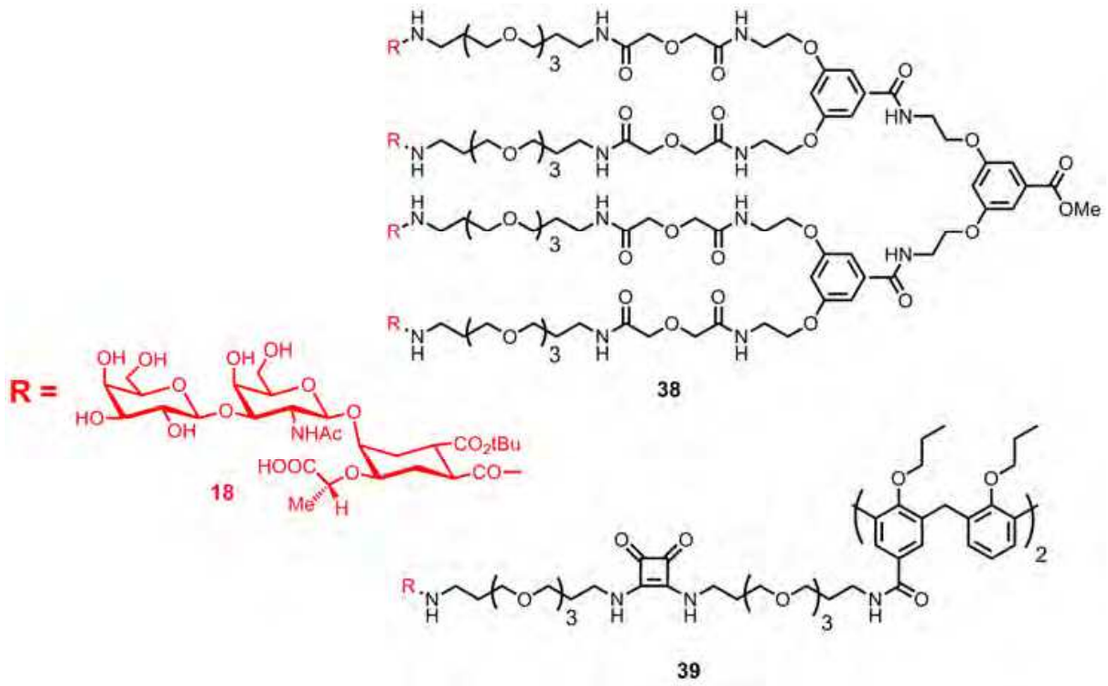


Fig. 18. A scheme of multivalent Bernardi-Pieters' inhibitor based on dendridic structure and Bernardi-Casnati's inhibitor based on Calix-[4]-arene core.

The most recent example of using multivalent strategy in the design of Cholera toxin inibitors presented in this chapter is based on the work of Tran and collaborators (Tran, Kitov et al. 2011). They are intensively working on designing a bidentate multivalent ligands. In their recent work they describe the synthesis and activities of a series of galactose conjugates on polyacryl amide and dextran. Nanomolar affinity of inhibitors against CT was obtained by conjugation of a second fragment (corresponding Neu5Ac's mimic), while galactose-only progenitors showed no detectable activity. The general idea of such inhibitor's design is shown in Fig. 19.

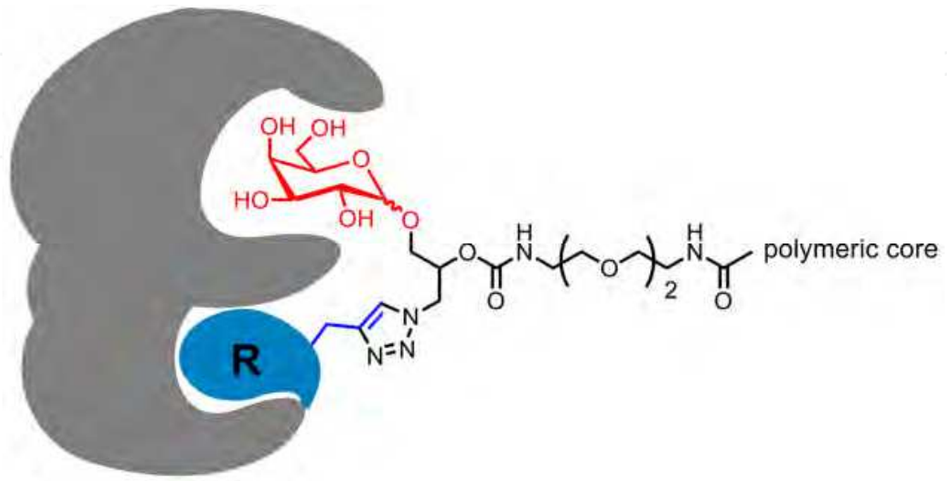


Fig. 19. Scheme for general design of multivalent Tran-Kitov's bidentate inhibitors.

A variety of multivalent inhibitors were described in this chapter. Basically, the multivalent inhibitors are designed using linkers to connect a galactose anchor to polymeric or dendrimeric cores or symmetric cores (5-fold symmetry). Recently, bidentate multivalent inhibitors were designed with conjugation of second fragment of that corresponds to Neu5Ac mimic. Generally, the above results showed that strategy of designing multivalent presentations of monovalent ligands can bring affinity closer to what is required for practical application against CT.

6. Novel binding site for blood group antigens in Cholera toxin: Potential target for the design of new Inhibitors?

At the end of the 1970s, two epidemiological studies established a dependency between the severity of Cholera infections and the blood group phenotype (Baura, Paguio 1977; Chaudhuri, De 1997). In these studies it was reported that people with blood group O were more prone to develop severe symptoms in comparison with people of blood group A, B or AB phenotype. Also, it was found that this dependency is strain specific, for example, in *V. cholerae* O1 "El Tor" (responsible of the seventh (current) pandemic) and *V. cholerae* O139 infections a connection with blood group phenotype of individuals was found (Glass et al. 1985, Swerdlow et al. 1994, Farruque et al. 1994, Tacket et al. 1995, Harris et al. 2005, Harris et al. 2008). On the other hand, for infection with classical *V. Cholerae* strains, no such association was observed.

The blood group phenotype of an individual is determinated by the presence or absence of antigenic substances on the surface of red blood cells. The ABO antigens are fucosylated oligosaccharide structures, carried on both glycolipids and glycoproteins. (Fig. 20) These antigens are not only on the surface of red blood cells, but are widely distributed throughout body fluids and tissues and are found also in the small intestine, the site of Cholera and ETEC infections. In this tissue, blood group antigens are presented on the intestinal epithelium cell surface (Finne 1989, Breimer 1984, Björk 1987), close to GM1 gangliosides. Structurally, ABO antigens are very similar, the H antigen (responsible of the O phenotype) is a tetrasaccharide characterized by a terminal fucose residue. The A and B antigens are pentasaccharides with a core similar to the H antigen, but each contain and additional saccharide residue- a terminal 2'-*N*-acetyl galactosamine (GalNAc) in the A antigen, or a terminal galactose for the blood group B antigen.

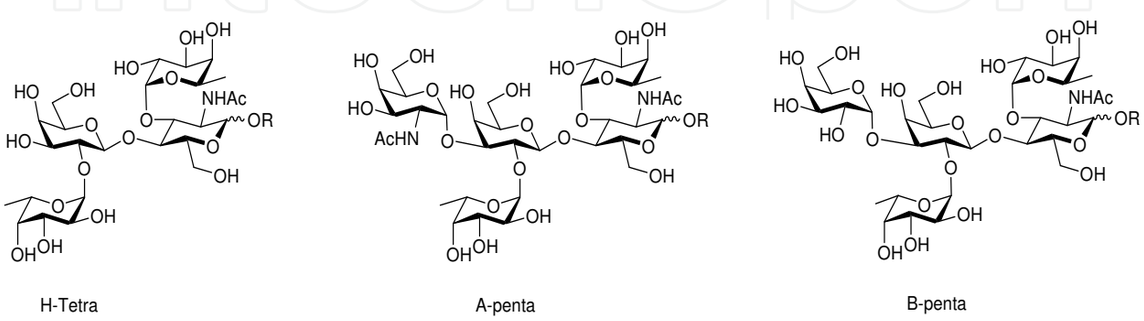


Fig. 20. Schematic representation of the Blood Group antigens H, A and B.

Following the discovery of the relation between blood group phenotype and cholera susceptibility, many studies have been conducted in order to investigate the ability of cholera toxin and of the highly homologous heat-labile enterotoxin (LT) from enterotoxigenic Escherichia coli (ETEC), to recognize blood group antigens of the ABO system (Bennun 1989, Monferran 1990, Barra 1992, Balanzino 1994, Balanzino 1999). It has been hypothesized that blood group antigens, with a preference for A and B epitopes, might disturb the action of the toxin by interfering with binding to GM1 ganglioside in the small intestine. However, the well-conserved GM1 binding site of CT is believed to be ganglioside-specific and cannot accommodate the fucosylated blood group antigens according to computer modelling. Consequently, the basis for the recognition of blood group antigens by Cholera Toxin at a molecular level is still unclear. In a recent investigation, Teneberg and co-workers have discovered a novel carbohydrate binding site studying a chimera between the B subunits of cholera toxin and the E. coli LT. This CTB/LTB chimera was shown to bind blood group A or B antigens on type 2 chains (Ångström 2000), and was subsequently characterized in complex with a blood group A analogue using protein crystallography by Krengel and co-workers (Holmner 2004). The structure of such complex is shown in Fig 21a. A follow-up study showed that native LTB, despite binding blood group antigens with lower affinity, also display the same mode of binding as the CTB/LTB chimera (Holmner 2007). In both cases, this binding site for blood group ligands is clearly distinct from the primary GM1 binding site. The blood group recognition site is located at the interface of two B-subunits, with one of the 2 subunits providing the majority of the contacts to the ligand. Based on the two crystal structures, it was possible to explain how the toxins discriminate between different ABH epitopes. The GalNAcα3 residue characteristic of blood group antigens binds with the toxin via several hydrogen bonds, including one involving its acetamido nitrogen (Fig 21b). The blood group B antigen is characterized by a terminal galactose residue and only differ from the A antigen at the 2-position, i.e. the acetamido group is replaced by a hydroxyl group. This hydroxyl group should preserve most of the interactions with the toxin and explains why the toxin does not discriminate notably between A and B epitopes. The fucose residue on the ABH antigens is also an important contributor to receptor recognition, however, blood group H determinants lack the entire terminal saccharide residue compared to blood group A and B determinant, and would therefore be expected to have significantly reduced binding affinities to cholera toxin. This assumption is substantiated by the finding that the loss of a single water-mediated hydrogen bond to the terminal GalNAcα3 residue results in a pronounced decrease in binding affinity (Holmner 2004), confirming the importance of the terminal GalNAc residue characteristic of blood group A antigens in molecular recognition. All these new contributions to understand the molecular basis of the interaction between blood group antigens and cholera toxin were reviewed in more detail by Krengel and co-workers (Holmner 2010).

In conclusion, the new information on the molecular recognition of blood-group antigens by Cholera Toxin should encourage medicinal chemist to development improved drug design strategies to prepare new pharmacological agents that inactivate cholera toxin. Inhibitors of the interaction of the cholera toxin with its primary receptor, the GM1 ganglioside, are especially attractive. Development of antagonists for the blood group

binding site of cholera toxin could enable a more effective combined therapy together with GM1 antagonists and, furthermore, could be used as a tool to understand the variability of susceptibility to Cholera infection with the blood group phenotype of individuals.

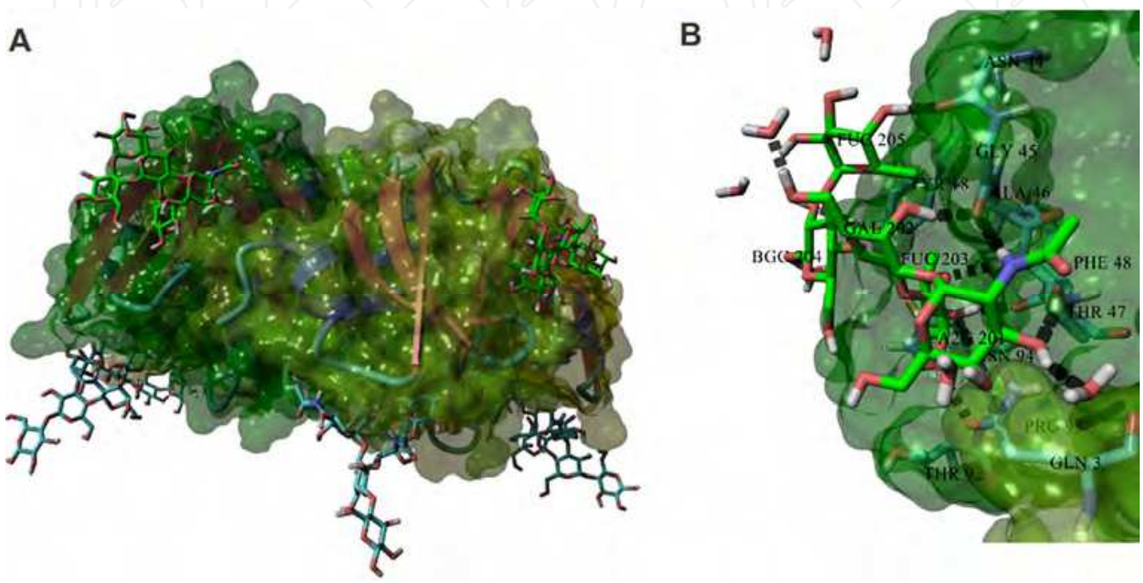


Fig. 21. (A) A crystallographic representation of CTB/LT chimera that shows the comparison between the binding site of blood group A pentasaccharide analog (green sticks) and GM1 ganglioside (sky-blue sticks) superimposed for comparison. (B) Interactions between blood group A and CTB/LT chimera – close view (Holmner 2010).

7. Methods and software

The methodology of structure based design of CT is described in several occasions (Podlipnik and Bernardi 2007; Zhang 2009). Structure based design starts with preparing a model of the protein receptor site. Models described in our review are based on receptor:ligand complex. In case of exploring of A-site ligands (Section 3) we have used a model based on crystallographic structure of CTA_1:ARF_6-GTP complex (PDB-ID: 2A5F)(Hol, O'Neal et al. 2005). A high resolution crystallographic structure (1.25 Å) of Cholera toxin B pentamer complexed with oGM1 (PDB-ID:3CHB) (Merritt, Kuhn et al. 1998) has been used as a template for generating a model in the case of exploring GM1 mimics (Section 4). The raw crystallographic structures were in both cases optimized with protein preparation wizard provided as part of the Schrodinger Suite 2011 (http://www.schrodinger.com). The interaction field grids that were used for docking were centered at the center of the ligand

(NAD^+ in case of CT's A-Site; oGM1 in case of B-Site). All ligands described in Sections 3 and 4 are prepared using Schrodinger's Ligprep. Glide XP (Murphy, Repasky et al. 2011) has been used for docking. The figures 1,2,5,7,9,12,14 and 21 representing poses were perpared with YASARA (http://www.yasara.com).

8. Conclusions

We reviewed different strategies to design an effective cure against cholera infections. The first strategy is based on exploring natural ligands as potential inhibitors of the ADP-ribosylation function of CT A subunit. The data collected from various sources indicate that catechin derivatives found in different natural sources could limit enzymatic activity of CT. Maybe this is one of the major reasons why during the centuries cholera pandemies have spared China and Japan, the catechin-consumig countries. The second approach is to design mimics in a mono- and/or multivalent presentation that could bind to the GM1 binding site in the B-pentamer, and thus prevent binding to GM1 receptors at the surface of epithelial cells, the first act necessary for Cholera toxin intoxication. The rational design of GM1 mimics is one of the most representative example of using structural information supported by molecular modelling methods in task to get an effective inhibitor. Nice examples of how multivalent presentation of single ligands can enhance affinity to CT by several orders of magnitude, and thus reach the levels of affinity required for practical applications against CT were presented. In addition we introduced a new strategy for developing CT inhibitors by targeting a newly identified binding site for blood group antigens in CT. This chapter describes examples of some successful application of knowledge that is connected with molecular structures and processes at the molecular level to design inhibitors toward Cholera toxin. The challenge to transfer the knowledge described in our review to achieve the practical, economic and scalable preparation of CT inhibitors remains still open.

9. Acknowledgments

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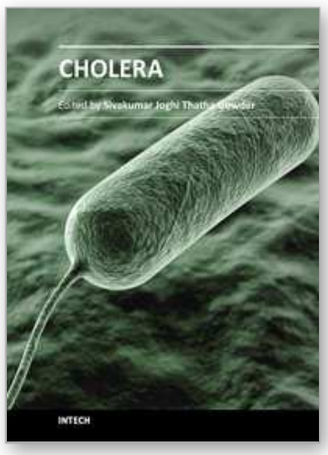
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Cholera, a problem in Third World countries, is a complicated diarrheal disease caused by the bacterium Vibrio cholerae. The latest outbreak in Haiti and surrounding areas in 2010 illustrated that cholera remains a serious threat to public health and safety. With advancements in research, cholera can be prevented and effectively treated. Irrespective of "Military" or "Monetary" power, with one's "Own Power", we can defeat this disease. The book "Cholera" is a valuable resource of power (knowledge) not only for cholera researchers but for anyone interested in promoting the health of people. Experts from different parts of the world have contributed to this important work thereby generating this power. Key features include the history of cholera, geographical distribution of the disease, mode of transmission, Vibrio cholerae activities, characterization of cholera toxin, cholera antagonists and preventive measures.

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