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TNO report

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Therapy after exposure to toxins (I)

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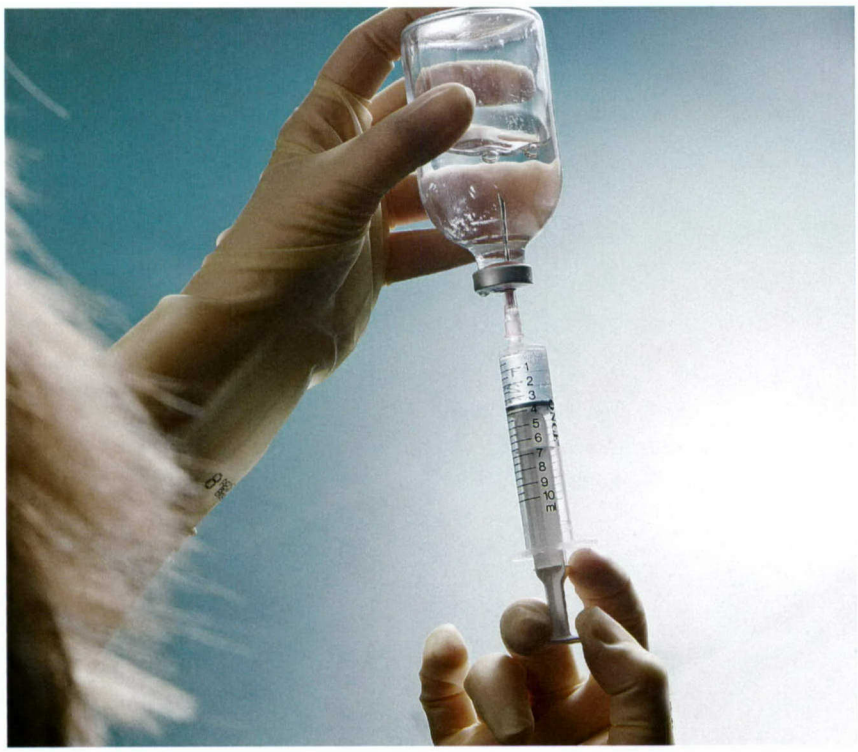
Therapie na blootstelling aan toxines (I)



Probleemstelling

Militairen kunnen tijdens hun werkzaamheden worden blootgesteld aan biologische agentia, waaronder toxines, met alle negatieve gevolgen van dien. Toxines kunnen in het lichaam worden geneutraliseerd door antilichamen. Het opwekken van deze antilichamen is echter veelal een kostbare en een bewerkelijke methode die weinig efficiënt is. Tevens zijn hierbij dieren nodig. Met behulp van een nieuwe methode, de SELEX-technologie (Systematic Evolution of Ligands by EXponential enrichment), kunnen hogeaffiniteit oligonucleotide liganden (soort antilichamen) worden verkregen. Hierbij wordt op een systematische wijze een goede binder (aptameer) geselecteerd uit een DNA-bibliotheek van circa 10^{14} mogelijke DNA-sequenties. Het doelmolecuul voor een aptameer kan een peptide (bijvoorbeeld ricine, cholera toxine, Stafylokokken Endotoxine B (SEB) of anthraxsporen) zijn of een niet-peptide (bijvoorbeeld dinitrotolueen of chloro-aromaten). Een aptameer is hiermee een soort alternatief antilichaam en heeft als voordeel dat de stabiliteit groter is dan die van de traditionele antilichamen. Een aptameer dat een toxine neutraliseert, kan potentieel als therapeuticum worden toegediend. Het doel van dit onderzoek is het nagaan of het mogelijk is om een aptameer tegen saxitoxine te ontwikkelen.

Deze aanpak wordt binnen de business unit Biologische en Chemische Bescherming van TNO Defensie en Veiligheid, locatie Rijswijk, onderzocht in het kader van het Programma V013 'Passieve bescherming tegen NBC-wapens' (resultaatnummer 809b). Van dit Programma is het Ministerie van Defensie de opdrachtgever.

Beschrijving van de werkzaamheden

Saxitoxine werd gekozen als modeltoxine, een militair relevant toxine maar ook een verbinding die mogelijk model kan staan voor toxische industriële chemicaliën (TIC's). Tevens werd een DNA-bibliotheek, bestaande uit een random sequentie dat aan beide zijden wordt geflankeerd door een bekende sequentie, ontwikkeld. In een later stadium zal vermeerdering van gebonden aptameren plaatsvinden door middel van Polymerase Chain Reaction (PCR). Hiervoor werden primers ontwikkeld op de twee bekende sequenties en de PCR-assay werd geoptimaliseerd.

Daarnaast werd een methode opgezet waarbij met behulp van magnetische bolletjes onderscheid kan worden gemaakt tussen goed bindende en niet bindende DNA-sequenties.

Resultaten en conclusies

De (Real Time) PCR van de DNA-bibliotheek werd geoptimaliseerd. Het gewenste product (dsDNA van 79 bp) kon worden gescheiden van eventuele bijproducten door het gebruik van een gel extractie kit. Uit dit dsDNA kan een ssDNA-product worden verkregen door het gebruik van streptavidine beads. Immobilisatie van een chemisch, niet-toxisch model voor saxitoxine aan magnetische bolletjes vond plaats met een een recovery van circa 80%. Op grond van de behaalde resultaten werd een protocol opgesteld voor de immobilisatie van saxitoxine aan dezelfde bolletjes.

Toepasbaarheid

Een aptameer dat een neurotoxine als saxitoxine kan binden, kan mogelijk als therapeuticum worden gebruikt om de saxitoxine uit het lichaam van een blootgestelde militair weg te vangen. Hierdoor zal de militair beter in staat zijn om zijn taak te vervullen.
De SELEX-technologie is daarnaast breed toepasbaar in ander onderzoek aan BC-wapens en TIC's (toxische industriële chemicaliën). In het kader van Detectie en Identificatie kunnen aptameren worden ontworpen voor bijvoorbeeld sensoren. Voor Diagnose en dosimetrie kunnen aptameren worden ontwikkeld die na koppeling aan bijvoorbeeld magnetische bolletjes kunnen worden gebruikt voor het isoleren van biomarkers uit monsters van menselijke oorsprong. Aptameren kunnen ook worden toegepast in ontsmettende middelen door het wegvangen van het target van de mens en uit het milieu.

Vervolgafspraken

Dit onderzoek zal verder worden voortgezet zoals vastgelegd in het Programma V013.

PROGRAMMA	PROJECT
Programmabegeleider A.S. de Koning, CDC/MGFB	Projectbegeleider Dr.H.J. Jansen, MGFB te Den Haag
Programmaleider Dr. Ir. M.S. Nieuwenhuizen, TNO Defensie en Veiligheid, Business Unit Biologische en Chemische Bescherming	Projectleider Dr. M. Polhuijs, TNO Defensie en Veiligheid, Business Unit Biologische en Chemische Bescherming
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Frequentie van overleg Met de programma/projectbegeleider werd meerdere malen gesproken over de invulling en de voortgang van het onderzoek.	Projectteam Dr. M. Polhuijs, A.M.B.C. de Vries, ing. W.E. Kaman, Ir. M.P. Broekhuijsen, Dr. P.A.V. van Hooft, Dr. D. Noort, TNO Defensie en Veiligheid

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Summary

During their activities, military personnel can be exposed to biological agents, amongst them toxins. If exposure has occurred, efficient therapy with, for instance, an antidote is needed. Toxins can be neutralised in the body with antibodies. However, the development of antibodies is a time-consuming and laborious procedure. In addition, it involves the use of animals. Recently, articles appear in literature in which alternative antibodies, aptamers, are developed by means of the SELEX technology (Systematic Evolution of Ligands by EXponential enrichment). This method seems a more simple method than the development of antibodies.

SELEX is a method for the production of specific, high affinity oligonucleotide ligands against target molecules [Gold, 1995; Gold et al., 1997; Iqbal et al., 2000; Toulmé, 2000]. The target molecule can either be a peptide as, for instance, ricin [Hesselberth et al., 2000], cholera toxin [Bruno and Kiel, 2002] and SEB [Bruno and Kiel, 2002] or anthrax spores [Bruno and Kiel, 1999]. Aptamers against non-peptides could be developed as well: DNT [Bruno, 1997a] or chloroaromates [Bruno, 1997b]. The aim of this work is to study whether it is possible to develop aptamers against the model toxin saxitoxin that can be used for therapeutic purposes. As a first approach, the feasibility of the development of aptamers was studied.

Preliminary results show that it is feasible to proceed with the development of aptamers as therapeutic tools. It is anticipated that the optimized PCR method, together with the developed DNA library and the corresponding primers can also be used for the development of aptamers against other ligands as well. The SELEX protocol with both positive and negative selection rounds will be designed. Based on the results with a chemical, nontoxic model compound of saxitoxin (STX), it is anticipated that STX can be immobilized on magnetic beads. This complex will be mixed with a 5-fold excess of DNA library at room temperature (37 °C in some rounds). Bound DNA will be removed from the magnetic beads, followed by amplification with PCR. Product will be separated on gel and purified with a Gel extraction kit. The biotinylated PCR product will be separated into ssDNA by means of streptavidin beads. The ssDNA that was originally not coupled to biotin, which is an exact copy of the original bound DNA, will be amplified by PCR. This amplified product will be added to the immobilized STX in a subsequent round of SELEX.

An aptamer that can bind a neurotoxin as saxitoxin might be used as a therapeutic compound to neutralize and remove the toxin from the body of exposed military personnel. This would contribute to fulfilling one of the main objectives of the Ministry of Defence in the area of 'Passive NBC Protection', i.e. 'Protection of personnel against adverse health effects as a result of (non intentional) exposure to biological agents under operational circumstances'.

Contents

1 Introduction

During their activities, military personnel can be exposed to biological agents, amongst them toxins. If exposure has occurred, efficient therapy with, for instance, an antidote is needed. In the past, research was performed to design and study peptides that would block the binding site for the neurotoxins tetrodotoxin en saxitoxin at sodium channels in the brain without affecting the functional activity of those channels. A first approach was to design antidotes via combinatorial synthesis of analogues of μ-conotoxin with an antagonistic effect [Rumley-van Gurp et al., 1998]. With the second approach, antidotes were produced by means of phage display [Broekhuijsen et al., 2000]. Both studies did not result in antidotes that could bind to the sodium channel without loss of functional activity.

Another strategy would be the use of antibodies against the toxin itself. Preparing antibodies is a laborious and time-consuming method with a low efficiency. In addition, the use of animals is required. An alternative method to prepare antidotes would be phage display. But, the use of this method did not result in good candidates in the past. One explanation would be the fact that the method was just set-up at that time. Recently, articles appear in literature in which antibodies are developed by means of the SELEX technology (Systematic Evolution of Ligands by EXponential enrichment). This method seems a more simple method than phage display.

SELEX is a method for the production of specific, high affinity oligonucleotide ligands against target molecules [Gold, 1995; Gold et al., 1997; Iqbal et al., 2000; Toulmé, 2000]. A random pool of oligonucleotides, flanked by known sequences, is brought into contact with a target molecule. Oligonucleotides with a high affinity for the target will bind to that molecule. Unbound oligonucleotides are separated from the bound oligonucleotides. Subsequently, the bound oligonucleotides can be released from the target and amplified by means of Polymerase Chain Reaction (PCR). This whole process can be repeated several times with amplified oligonucleotides from the former cycle. This procedure will result in the selection of an aptamer, a DNA or an RNA sequence that is capable of binding with a high affinity to the target molecule.

The target molecule can either be a peptide as, for instance, ricin [Hesselberth et al., 2000], cholera toxin [Bruno and Kiel, 2002] and SEB [Bruno and Kiel, 2002] or anthrax spores [Bruno and Kiel, 1999]. Aptamers against non-peptides could be developed as well: DNT [Bruno, 1997a] or chloroaromates [Bruno, 1997b]. The aim of this work is to study whether it is possible to develop aptamers against the model toxin saxitoxin that can be used for therapeutic purposes. As a first approach, aptamers will be developed.

2 Materials and Methods

2.1 Biological materials

The restriction enzymes EcoR I en Hind III were from BioLabs New England.

2.2 Chemicals and materials

The synthetic oligonucleotide primers were obtained from Isogen, Bioscience B.V. or Qiagen. The DNA template was procured from Isogen, Bioscience B.V.. The Superladder-Low 20bp Ladder (50 bands: 20bp-1000bp in exact 20bp increments, brighter bands at 200bp and 500bp) were from Eurogentec. Taq DNA Polymerase PCR buffer, Mg2+, deoxynucleoside triphosphates, SybrGreen and FastStart SybrGreen were from Roche. Low Agarose Gel and 15% TBE-Urea PAGE gels were from BioRad. The Gel Extraction Kit was from Qiagen.
Saxitoxin diacetate was from Sigma. MPG Long Chain Alkylamine magnetic beads (5 μm, 50 nm pore diameter) were from CPG Lincoln Park, NJ, USA.

2.3 Primer design

Primer design, DNA sequence manipulation and choice of restriction enzymes were performed with DNAMAN 4.11, Lynnon BioSoft. Two set of primer pairs were developed and tested.

Short primer pair
Primers with a length of 20 bp, a GC content of circa 40-60% and Tm 60 – 61 °C were designed. Primers had to contain internal restriction sites for cloning the aptamers in a pUC19 vector.
F-Eco-biotin (Isogen) was the first biotinylated forward primer tested: biotine-5'-GCGAATTCTAGGCATGCACG-3', containing an internal EcoR I restriction site (GAATTC).
And R-Hind-biotin (Isogen) was the first biotinylated reverse primer tested: biotine-5'-GCAAGCTTCCTCCAGGTGAC-3', containing an internal Hind III restriction site (AAGCTT).

Longer primer pair
Primers with a length of circa 25 bp, a GC content of circa 40-60% and Tm 60 – 61 °C were designed. Primers had to contain internal restriction sites for cloning the aptamers in a pUC19 vector.
Forward primer (F-Eco2-biotine: Isogen):
biotine-5'-CACACGAATTCTAGGCAATACAACA-3', containing an internal EcoR I restriction site (GAATTC); 25 bp, 40% GC, T=60.2 °C.
Reverse primer (R-Hind2-biotine; Isogen):
biotine-5'-GTCAAAAGCTTCATCCAGGATAGG-3', containing an internal Hind III restriction site (AAGCTT); 24 bp, 45.8% GC, T= 59.6 °C.
F-Eco3 (Isogen) and F-Eco4 (Qiagen) differed from F-Eco2-biotine in that they were not biotinylated. The same applied to R-Hind 3 (Isogen) and R-Hind 4 (Qiagen).
The sequence of R-Hind5-bio (Qiagen) is identical to that of R-Hind2-biotine (Isogen).

2.4 DNA library

Two DNA libraries (ssDNA) were used, containing fixed sequences for primer annealing in a PCR amplification reaction. The first starting nucleic acid ligand library was composed of 60-mers, containing a random sequence of 20 nucleotides (N20) attached to 5' and 3' fixed primer annealing sequence with a length of 20 oligonucleotides each (Apta20; Isogen): 5'- GCGAATTCTAGGCATGCACG-N20-GTCACCTGGAGGAAGCTTGC-3'.
A second starting nucleic acid ligand library that was used, was composed of 79-mers, containing a random sequence of 30 nucleotides (N30) attached to 5' and 3' fixed primer annealing sequence with a length of 25 and 24 oligonucleotides, respectively (Apta30; Isogen): 5'-CACACGAATTCTAGGCAATACAACA -N30-CCTATCCTGGATGAAGCTTTTGAC-3'
In addition, one sequence from the second DNA library was chosen en ordered from Qiagen (Apta30enkel):
5'-CACACGAATTCTAGGCAATACAACAAATTATGAGAACAGGGACCGCAC GTATCTCCCTATCCTGGATGAAGCTTTTGAC-3'

2.5 PCR amplification

PTC200 PCR from Biozym or the primus 96 from Peqlab
PCR amplification was performed with 2.5 U of Taq polymerase, a variable amount of library DNA (9 fg-1.2 µg), appropriate primers (0.1-1 µM concentration of each; +/- biotinylated), a 0.2 mM concentration of each deoxynucleoside triphosphate, and MgCl2 (1-6 mM) in a PCR volume of 50 µl.
Amplification was started with an initial cycle of 5 min denaturation at 95 °C. Followed by 20-40 cycles, each consisting of 30 sec denaturation at 95 °C, 30 sec of annealing at 60-70 °C, and 30 sec of polymerase elongation at 72 °C. After these cycles, temperature was kept at 72 °C during 5 min in order to allow complete elongation of all product DNA, followed by cooling to 15 °C. Amplification products (10 µl PCR product) were visualized by Low Range Agarose gel electrophoresis (2 or 3% wt/vol agarose; 200 V/100 mA, t =45-60 min; equipment from BioRad and power supply from Pharmacia) and ethidium bromide staining to confirm the sizes of the products, unless described otherwise. The Superladder-Low 20bp Ladder (50 bands: 20bp-1000bp in exact 20bp increments, brighter bands at 200bp and 500bp) was used.

Real Time PCR
Real Time PCR amplification was performed in the LightCycler with a variable amount of library DNA (9 fg-1.2 µg), appropriate primers (0.5 µM concentration of each), MgCl2 (1-4 mM) and 2 µl SybrGreen in a PCR volume of 20 µl.
Amplification was started with an initial cycle of 5 min denaturation at 95 °C. Followed by 21-41 cycles, each consisting of, if necessary a rapid heating with 20 °C/min to 95 °C, 5 sec of annealing at 60 °C, and 30 sec of polymerase elongation at 72 °C. After these cycles, temperature was kept at 72 °C during 1 min in order to allow complete elongation of all product DNA, followed by the melting program.

Real Time PCR in combination with Fast Start
Real Time FastStart PCR amplification was performed as for the normal Real Time PCR with 4 mM MgCl2 and 2 µl FastStart SybrGreen. The primer pair F-Eco4/ R-Hind5-bio was used.

Amplification was started with an initial cycle of 10 min denaturation at 95 °C. Followed by 45 cycles, each consisting of 10 sec denaturation at 95 °C, 5 sec of annealing at 60 °C, and 5 sec of polymerase elongation at 72 °C. After these cycles, temperature was kept at 72 °C during 30 sec in order to allow complete elongation of all product DNA, followed by the melting program.

2.6 Separation of dsDNA into ssDNA

The DNA library (12 ng) and the control ssDNA sequence (12 ng) were amplified by means of PCR with the F-Eco4/ R-Hind5-bio primer pair. The product formed was loaded on a 3% Low Range Agarose gel, followed by electrophoresis. The product of 79bp was extracted with a Gel Extraction Kit according to the manufacturer's instructions. Subsequently, the ssDNA's were isolated with streptavidin magnetic beads. Various amounts of beads, ranging from 100-600 µg were used. The original dsDNA, which was retained through the biotinylated complementary strand, was denatured according to the manufacturer's protocol. The ssDNA that was coupled to the magnetic beads was isolated, taken up in water and uncoupled by heating the beads during 5 min at 95 °C. The ssDNA that was not coupled to the magnetic beads was purified by ethanol precipitation after the addition of 10 µl of an aqueous solution of sodium hydroxide (0.1 M). Subsequently, both isolated ssDNA fractions were amplified with PCR (29 cycles). The amplified products were loaded on a 3% Low Range Agarose gel, followed by electrophoresis. Both the ssDNA fractions were also loaded on a 15% TBE-Urea PAGE gel, followed by electrophoresis. This time, the products were visualised by staining with Sybr Gold.

2.7 Immobilisation of saxitoxin on magnetic beads

For the reaction between between saxitoxin diacetate and the NH2-group of the magnetic beads a coupling method as described in literature [Huot et al., 1989; Kralovec et al., 1996; Micheli et al., 2002] was modified. The reaction between saxitoxin diacetate and the magnetic beads was first studied with a non-toxic chemical model compound of saxitoxin, phenylcarbamate. This compound (1.66 mg; 12.1 µmol) and the magnetic beads (2.4 µmol amino functions; washed several times with 100 µl of water) were added to 200 µL of ethanol. To this mixture, a 5-fold excess of formalin and a catalytic amount of hydrochloric acid were added. Hereafter the mixture was incubated during 7 days under continuous shaking. The reaction mixture was filtered over a 3kD filter and the eluate was evaporated. The concentration of phenylcarbamate before the addition to the magnetic beads as well as in the residue of the eluate was determined with 1H-NMR.

3 Results

3.1 Amplification of the DNA library

3.1.1 PCR

A 50 µl PCR master mixture consisting of library DNA, 0.5 µM of each primer (F-Eco4 and R-Hind5-bio), 0.2 mM dNTPs, 1.5 mM $MgCl_2$ and 2.5 U of Taq polymerase appeared to provide the best results. Mostly, the lowest amount of DNA in the PCR assay for a good amplification product had to be in the range of 1 ng. Optimal PCR conditions were determined to be initial denaturation at 95 °C for 5 min, 40 cycles of 95 °C for 30 sec, 70 °C for 30 sec and 72 °C for 30 sec and a final extension at 72 °C for 5 min.

Amplification products were visualized by Low Range Agarose gel electrophoresis (200 V/100 mA, t =60 min) and ethidium bromide staining to confirm the size of the products. When using 3% wt/vol agarose gel, the separation of the amplified products was superior to that when using a 2% wt/vol agarose gel.

When using these conditions, a clear, and a relatively clean amplification product of the expected molecular weight of 79 bp was obtained when using 120-1.2 ng DNA library (Figure 1, lanes 2, 3 and 4, respectively). Lower amounts of 120 and 12 pg DNA in the PCR assay, did not result in a clear amplification product (Figure 1, lanes 5 and 6, respectively). Similar results were obtained with the selected sequence from the Apta30 library, Apta30enkel (Figure 1, lanes 7-11 with 120 ng, 12 ng, 1.2 ng, 120 pg and 12 pg, resp). Blanc sample, to which no DNA was added (Figure 1, lane 12), did not result in an amplification product.

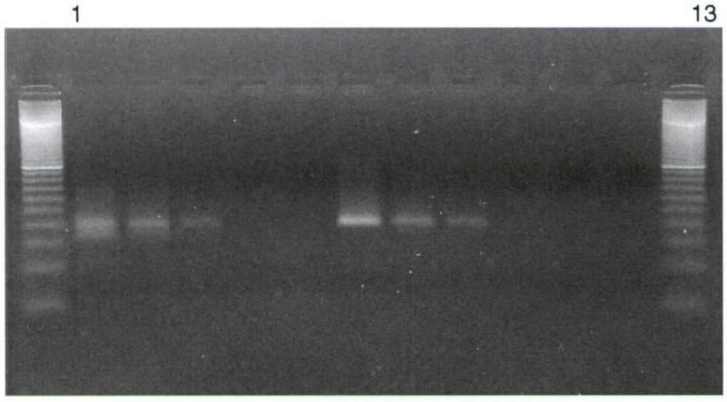


Figure 1 PCR amplification was performed with the DNA library Apta30 (40 cycles) with different amounts of library DNA: 120 ng (lane 2), 12 ng (lane 3), 1.2 ng (lane 4), 120 pg (lane 5) or 12 pg (lane 6). Results were compared with the amplification product of the selected sequence from the Apta30 library (Apta30enkel; 40 cycles) with different amounts of Apta30enkel DNA: 120 ng (lane 7), 12 ng (lane 8), 1.2 ng (lane 9), 120 pg (lane 10) or 12 pg (lane 11). A blank assay to which no DNA was added was performed as well (lane 12). Primers used were F-Eco4 and R-Hind5-bio. Lanes 1 and 13 are the Super-ladder-Low 20bp Ladder (50 bands: 20bp-1000bp in exact 20bp increments, brighter bands at 200bp and 500bp).

3.1.2 Real Time PCR

Real Time PCR amplification was performed in the LightCycler with 0.5 µM concentration of each primer (F-Eco4 and R-Hind5-bio), 4 mM $MgCl_2$ and 2 µl SybrGreen in a PCR volume of 20 µl. Mostly, the lowest amount of DNA in the PCR assay for a good amplification product had to be in the range of 1 ng.
Amplification was started with an initial cycle of 5 min denaturation at 95 °C. Followed by 31 cycles, each consisting of, if necessary a rapid heating with 20 °C/min to 95 °C, 5 sec of annealing at 60 °C, and 30 sec of polymerase elongation at 72 °C. After these cycles, temperature was kept at 72 °C during 1 min in order to allow complete elongation of all product DNA, followed by the melting program.
PCR assays with 21 PCR cycles containing the control aptamer, resulted in a melting peak at 81 °C (Figure 2, third panel, blue or green line). The blanc assay showed almost no product under the same PCR conditions (Figure 2, third panel, red line). Increasing the number of cycles to 41 resulted in similar results in case of the DNA library (Figure 2, lower panel, blue or green line). In the blanc assay, a peak with melting point 71 °C appeared (Figure 2, lower panel, red line).
In the assays to which the DNA library was added, a melting peak at 81 °C appeared as well (Figure 2, upper panel, blue or green line), although with a lower fluorescence signal in comparison to the control aptamer. In addition, already after 21 cycles, the peak with a melting point at 71 °C appeared as well (Figure 2, upper and second panel, blue or green line).

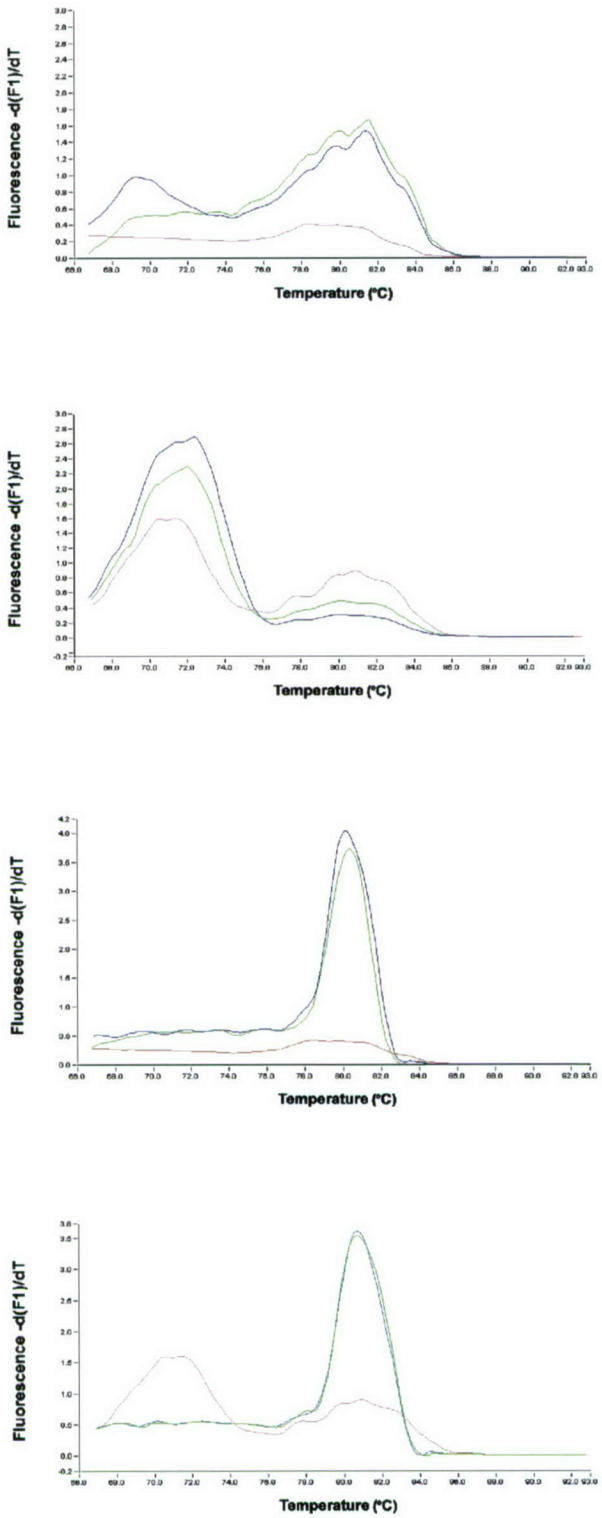


Figure 2 Real Time PCR was performed with the DNA library Apta30 (blue line: 120 pg; green line: 1.2 pg) with different amounts of PCR cycles: 21 (upper panel) or 41 (second panel). Results were compared with the amplification product (blue line: 120 pg; green line: 1.2 pg; 21 cycles in third panel and 41 cycles in the lower panel) of the selected sequence from the Apta30 library (Apta30enkel). A blank assay to which no DNA was added was performed throughout all experiments (red line in all panels). Primers used were F-Eco4 and R-Hind5-bio.

The samples from the Real Time PCR were run on a 3% agarose gel as well. The selected sequence, Apta30enkel, was amplified into a product of 79 bp (Figure 3, lanes 5 and 6). A similar product was observed when the DNA library was amplified under the same conditions with 21 cycles (Figure 3, lane 1). But, in addition to this product a smear was present which could not be detected in the case of amplification of the selected sequence. With an increasing amount of PCR cycles, it seemed as if the desired PCR product diminished with a concurring increase of the higher molecular weight products in the smear (Figure 3, lanes 2-4). Under all conditions tested, amplification of a blank assay to which no DNA was added, also a product of 79 pb was formed (Figure 3; lanes 8-11).

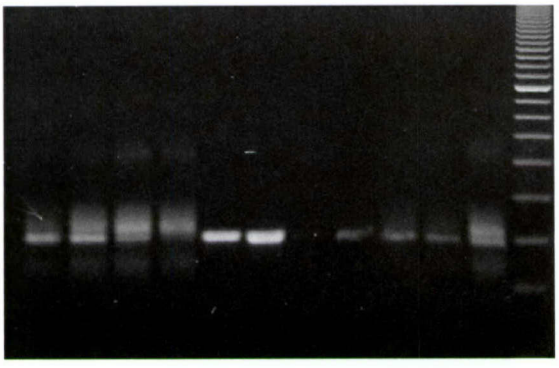


Figure 3 Real Time PCR was performed with the DNA library Apta30 (an absolute amount of 120 pg DNA in the PCR assay) with different amounts of PCR cycles: 21 (lane 1), 31 (lane 2), 36 (lane 3) or 41 (lane 4). Results were compared with the amplification product of the selected sequence from the Apta30 library, (Apta30enkel with an absolute amount of 120 pg DNA in the PCR assay: 21 cycles in lane 5 and 41 cycles in lane 6). A blank assay to which no DNA was added was performed as well (12 cycles, lane 8; 31 cycles (lane 9), 36 (lane 10) or 41 (lane 11). Primers used were F-Eco4 and R-Hind5-bio. In lane 7, no sample was added. Lane 12 is the Super-ladder-Low 20bp Ladder (50 bands: 20bp-1000bp in exact 20bp increments, brighter bands at 200bp and 500bp).

3.1.3 Real Time PCR FastStart

The FastStart procedure was performed as described in Paragraph 2.5 under 'PCR amplification'. This procedure can be used to prevent or minimize the formation of primer-dimers and other by-products. With 21 PCR cycles, the blank assay to which no DNA was added, did not show primer-dimers or other by-products (Figure 4, lane 8). PCR amplification of the selected control sequence under similar conditions resulted in an expected product of 80 bp when an absolute amount of 12 ng was used in the PCR assay (Figure 4, lane 6). When 120 pg DNA was added, a product with a higher molecular weight was formed (Figure 4, lane 7). Results for the amplification of the DNA library were opposite to those of the selected control sequence. When an absolute amount of 1.2 pg of the DNA bank was amplified, the desired product of 79 bp was formed (Figure 4, lane 5). When using higher amounts of DNA ranging from 120 pg to 1.2 µg, a product with a higher molecular weight was formed (Figure 4, lanes 2-5). It seems as if different products are formed under different PCR conditions. A similar phenomenon was observed in the results of the melting peaks as visualized in Figure 5. Obviously, the FastStart procedure did not provide unambiguously information on the identity of the various PCR products.

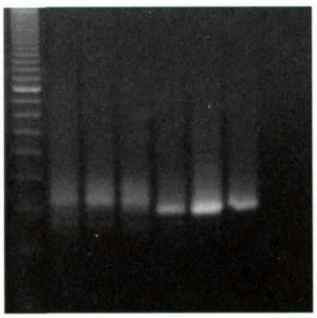


Figure4 Real Time PCR FastStart was performed with different absolute amounts of the DNA library Apta30 in the PCR assay with 21 PCR cycles (1.2 µg in lane 2; 12 ng in lane 3; 120 pg in lane 4; 1.2 pg in lane 5). Results were compared with the amplification product of the selected sequence from the Apta30 library (Apta30enkel; 12 ng in lane 6; 120 pg in lane 7; 21 cycles). A blank assay to which no DNA was added was performed as well (12 cycles, lane 8). Primers used were F-Eco4 and R-Hind5-bio. Lane 1 is the Super-ladder-Low 20bp Ladder (50 bands: 20bp-1000bp in exact 20bp increments, brighter bands at 200bp and 500bp).

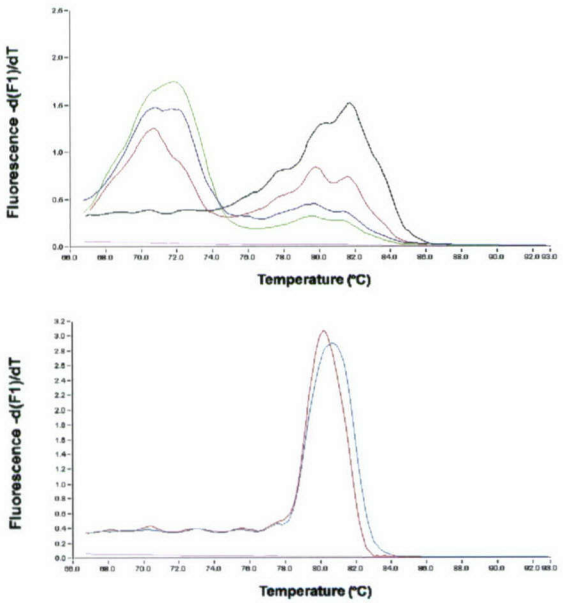


Figure 5 In the upper panel Real Time PCR with 12 cycles was performed with the DNA library Apta30 (green line: 1.2 µg; blue line: 12 ng; red line: 120 pg; black line: 1.2 pg; pink line: no DNA was added) Results were compared with the amplification product of the selected sequence from the Apta30 library (Apta30enkel) in the lower panel (blue line: 12 ng; red line: 120 pg). Primers used were F-Eco4 and R-Hind5-bio.

3.2 Separation of dsDNA into ssDNA with Streptavidin magnetic beads

Both the DNA library and the selected control sequence were amplified by means of a PCR assay (29 cycles) as described in Paragraph 3.1.1. The amplified products were loaded on a 3% Low Range Agarose gel, followed by electrophoresis. The expected product of 79bp was found both with the DNA library and the selected sequence. In addition, by-products were formed (data similar to Figure 1). The band containing the product of 79bp was isolated by means of the Gel Extraction Kit. The isolated dsDNA was separated into ssDNA's as described in Paragraph 2.6. Both isolated ssDNA fractions were amplified with PCR (Paragraph 3.1.1; 29 cycles) and the amplified products were loaded on a 3% Low Range Agarose gel, followed by electrophoresis.

The amplified products from this procedure in case of the DNA library are shown in Figure 6. Amplification of the ssDNA that was originally coupled to the magnetic beads resulted in the expected product of 79 bp (Figure 6, lanes 2-5). In addition, several products of higher molecular weight were formed. The effect of varying amounts of beads on the separation step was studied. In lane 2, 100 µg beads were added. For the lanes 3, 4 and 5 this was 200 µg, 400 µg and 600 µg, respectively. No large differences were observed. Results suggested that lower amounts of beads resulted in a more intense band of the expected product and a somewhat lower amount of high molecular weight by-products. The molecular weight of the product that was formed in the corresponding blanc assays was somewhat lower than the expected 79 bp (Figure 6, lanes 6-9; 100 µg beads in lane 6, 200 µg beads in lane 7, 400 µg beads in lane 8 and 600 µg beads in lane 9).
Amplification of the ssDNA that was originally not coupled to the magnetic beads resulted also in the formation of a product of 79 bp (Figure 6, lanes 10-13). This time, less products of higher molecular weight were formed. Again, the molecular weight of the product that was formed in the corresponding blanc assays was somewhat lower than the expected 79 bp (Figure 6, lanes 14-17). Varying amounts of beads did not influence the results.

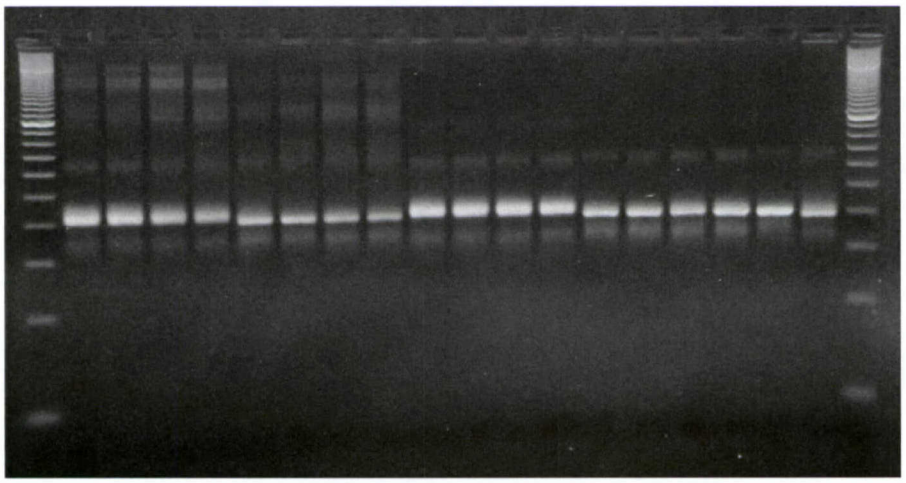


Figure 6 Separation of dsDNA from the DNA library into ssDNA by means of streptavidin magnetic beads, followed by PCR amplification of the ssDNA. Various amounts of beads of 100 µg (lane 2, 6, 10 and 14), 200 µg (lane 3, 7, 11 and 15), 400 µg (lane 4, 8, 12 and 16) and 600 µg (lane 5, 9, 13 and 17) were used. Amplification products of the ssDNA that was originally coupled to the magnetic beads (lanes 2-5) and those of the ssDNA that was originally not coupled to the magnetic beads (lanes 10-13) are present. The corresponding blanc assay to which no DNA was added are shown in the lanes 6-9 and lanes 14-17, respectively. Lanes 1 and 20 are the Super-ladder-Low 20bp Ladder (50 bands: 20bp-1000bp in exact 20bp increments, brighter bands at 200bp and 500bp). Products in the lanes 18 and 19 are not part of the experiments performed within the scope of this project.

The amplified products of the DNA library were loaded on a 3% Low Range Agarose gel, followed by electrophoresis as well (Figure 7). Results were comparable to those with the DNA library. Amplification of both the ssDNA that was originally coupled to the magnetic bead as well as the amplification of the ssDNA that was originally not coupled to the magnetic bead resulted in a similar product of 79 bp (Figure 7, lanes 4 and 5, respectively). Again, amplification of the ssDNA that was not coupled to the beads resulted in less by-products (Figure 7, lane 5) than assays in which the ssDNA that was originally coupled to the beads was amplified (Figure 7; lane 4). In these experiments only one amount of 200 µg of magnetic beads was used.

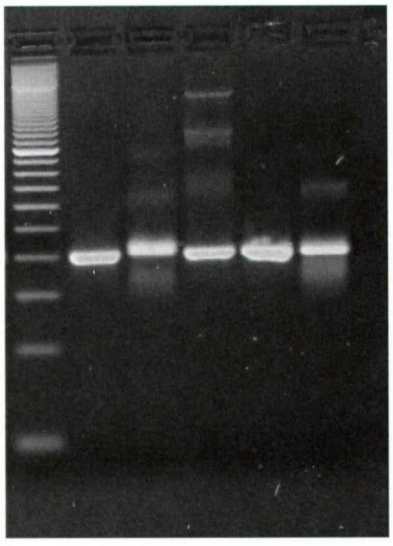


Figure 7 Separation of dsDNA from the selected sequence into ssDNA by means of streptavidin magnetic beads (200 µg), followed by PCR amplification of the ssDNA. The amplification products of the ssDNA that was originally coupled to the magnetic beads (lane 4) and those of the ssDNA that was originally not coupled to the magnetic beads (lane 5) are presented. For comparison, the amplification products of ssDNA from the selected sequence (lane 2; 12 ng) and the DNA library (lane 3; 12 ng) are presented as well. Lane 6 shows the results of the blanc PCR assay to which no DNA was added. Lane 1 is the Super-ladder-Low 20bp Ladder (50 bands: 20bp-1000bp in exact 20bp increments, brighter bands at 200bp and 500bp).

In order to study whether the abovementioned procedures indeed resulted into ssDNA, the ssDNA fractions were not amplified by PCR, but loaded on a PAGE gel, followed by electrophoresis. The ssDNA products visualized by Sybr Gold are shown in Figure 8. As a control, samples of the ssDNA library (Figure 8, lane 1, 120 ng; lane 2, 12 ng) and the selected ssDNA sequence (Figure 8, lane 3, 120 ng; lane 4, 12 ng) are shown as well. Clear bands were found at a similar height as the dsDNA product of 60bp in the Molecular weight ladder. Most likely, the ssDNA migration is different from that of the dsDNA products.

The library ssDNA that was originally not coupled to the magnetic bead (Figure 8, lane 7) also showed a clear product at the height of 60 bp. A similar band was found for the selected ssDNA sequence that was originally not coupled to the magnetic bead (Figure 8, lane 10).

The ssDNA that was originally coupled to the magnetic beads was processed in a heating step so that the ssDNA could be released from the magnetic bead. It was expected that these products would also appear as bands at the height of a 60 bp product. However, the library ssDNA showed a clear product at the height of circa 260 bp (Figure 8, lane 7). A similar phenomenon was observed in case of the selected ssDNA sequence (Figure 8 lane 10). It is most likely that after the heating procedure, the biotin and avidin were still coupled to the ssDNA strains.

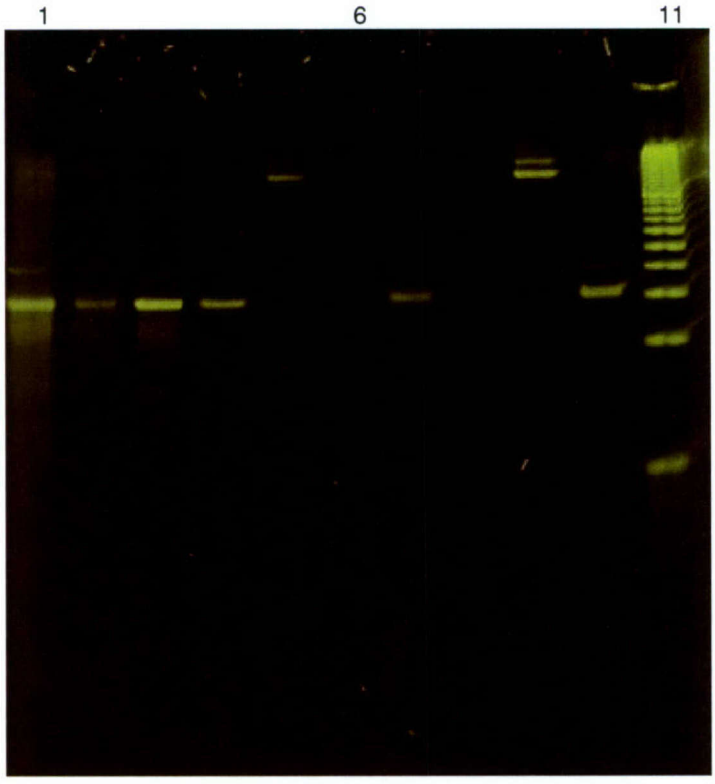


Figure 8 Visualization of the ssDNA products with Sybr Gold on a PAGE gel. As a control, samples of the ssDNA library (lane 1, 120 ng; lane 2, 12 ng) and the selected ssDNA sequence (lane 3, 120 ng; lane 4, 12 ng) are provided. The ssDNA that was originally coupled to the magnetic beads is shown in lane 5 (DNA library), lane 6 (blanc to which no DNA was added) and in lane 9 (selected sequence). The ssDNA that was originally not couples to the magnetic beads can be found in lane 7 (DNA library), lane 8 (blanc to which no DNA was added) and in lane 10 (selected sequence). Lane 11 is the Super-ladder-Low 20bp Ladder (50 bands: 20bp-1000bp in exact 20bp increments, brighter bands at 200bp and 500bp).

3.3 Immobilization of a model compound of saxitoxin on magnetic beads

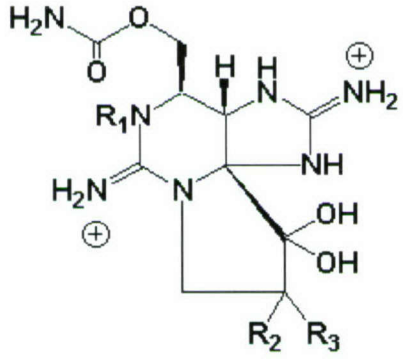
After a 7-days incubation of 12.1 μmol of the target phenylcarbamate with magnetic beads containing 2.4 μmol amino functions, 1.89 μmol target was immobilized on the magnetic beads as determined by 1H-NMR analysis of the remaining free phenylcarbamate (data not shown). Recovery, defined as % of the magnetic beads that is loaded with the target, was circa 80%. Based on this result, it was decided to prepare a protocol in order to perform a hot run with saxitoxin in the future.

4 Discussion and conclusions

During their activities, military personnel can be exposed to biological agents, amongst them toxins. If exposure has occurred, efficient therapy with, for instance, an antidote is needed. This report deals with the potential development of aptamers as antidotes, and as such can be used as therapeutics.

4.1 Choice of the model toxin

As a model toxin, saxitoxin (STX; Figure 9) was selected. This toxin is present on the various lists of threat agents, which makes it a military relevant toxin. Since several groups are already performing work in order to set up a therapy against intoxication with the more toxic botulism toxin, it was decided not to choose this toxin as a model toxin. An additional advantage of saxitoxin is that it is a non-peptide toxin. This means that it can also be used as a model compound for toxic industrial chemicals (TIC's).
In literature, data were found that indicated that it is possible to generate aptamers against small, non peptide molecules. Bruno and coworkers [1997b] demonstrated that it was possible to develop a mixture of aptamers with an affinity towards chloroaromatics. However, they did not determine the sequence or the affinity of the separate aptamers.



STX	R_1	R_2	R_3
STX	H	H	H
GTX-II	H	H	OSO_3^-
GTX-III	H	OSO_3^-	H
NeoSTX	OH	H	H
GTX-I	OH	H	OSO_3^-
GTX-IV	OH	OSO_3^-	H

Figure 9 Saxitoxin, paralytic shellfish toxin produced or elaborated by planktonic algae (dinoflagellates, in most cases) upon which the shellfish feed.

4.2 RNA or DNA aptamers

In earlier articles in literature, RNA aptamers were developed. Later on, also DNA aptamers were successfully used. Since DNA aptamers are more stable than RNA aptamers, it was decided to develop DNA aptamers.
Most work has been performed with ssDNA oligonucleotides, although some groups claim that dsDNA aptamers with high affinity can be developed as well [Bruno and Kiel, 2002]. It was decided to start the work with ssDNA aptamers.

4.3 Length of the random sequence of the DNA library and optimization of the PCR amplification step

Literature was studied in order to find a rationale for the length of the random sequence. However, no rationale was found; the impression was that in case of a smaller target, shorter aptamers were used.
It is generally accepted that decamers are already capable of forming loops. Nevertheless, longer sequences are probably more apt to form all kind of 3D structures. Therefore, a DNA pool with a random Section of 20 bp and two defined Sections of 20 bp each was used. When this DNA template of 60 bp was added to the PCR assay, a product could be visualized. But, it was not possible to obtain an amplification product without the formation of primer-dimers. Even the use of the LightCycler FastStart procedure did result in the formation of primer-dimers. Due to the very low molecular weight of the expected product that was almost similar to that of, for instance, primer-dimers, it was not possible to distinguish between the product and by-products and separate them efficiently with gel electrophoreses.
Therefore, a second DNA pool was constructed which consisted of 79 bp: a random sequence of 30 bp and two primer sequences of 25 and 24 bp, respectively. When using the newly developed primers, most of the time, negative blancs were obtained.
The new DNA-pool could be amplified with the corresponding primers into a product of the expected amount of base-pairs. Still, by-products were formed on some occasions. RealTime PCR was used in order to study the difference between the expected amplification product, the by-products and the products formed in the blank assays to which no DNA was added. However, the results did not provide unambiguously information on the identity of the various PCR products. In order to minimize the formation of primer-dimers and other by-products, the RealTime PCR with FastStart procedure was tested as well. Again, the results did not provide unambiguously information on the identity of the various PCR products. Therefore, optimal PCR conditions and conditions for an optimal separation of the desired product from the by-products were determined. An annealing temperature of 70 °C resulted in an optimal formation of desired product. The products could be separated on 3% Low Range Agarose gel from by-products very well. It was decided to incorporate a gel extraction of the desired product as a clean-up step in each SELEX round. Several methods were studied, but Qiagen Gel Extraction kit provided the best results.
It is anticipated that the optimized PCR method, together with the developed DNA library and the corresponding primers can also be used for the development of aptamers against other targets as well.

4.4 Separation of dsDNA into ssDNA

PCR amplified dsDNA (with a biotinylated reversed primer) was separated into ssDNA by means of streptavidin magnetic beads. The amount of magnetic beads was varied between 100 and 600 µg. The original dsDNA, which was retained through the biotinylated complementary strand, was denatured according to the manufacturer's protocol. Both isolated ssDNA strands, the one that was originally coupled to the magnetic beads as well as the ssDNA that was originally not coupled to the magnetic beads, could be purified very good. Varying the amount of magnetic beads had almost no effect on the results. In some experiments, results were slightly better with 200 µg. Since this was also the amount the manufacturer suggested, it was decided to use 200 µg of magnetic beads in further experiments. PCR amplification of both strands was satisfactory. However, it was decided to use the amplified product of the ssDNA

that was not coupled to the magnetic beads as an enriched DNA library in a following SELEX round. The use of this ssDNA has three advantages. First, these ssDNA's are exact copies of the original pools that were used. Consequently, the ssDNA that was originally coupled to the beads is a complementary strand. Secondly, amplification of the ssDNA that was originally not coupled to the magnetic beads resulted in a lower amount of high molecular weight by-products. Finally, it was not possible to release the biotin part from the complementary strand. It was possible to amplify the ssDNA from this larger product, but most likely, this procedure gave rise to the more undesired by-products.

4.5 Immobilization of saxitoxin on magnetic beads

The reaction between saxitoxin diacetate and the magnetic beads was first studied with a non-toxic chemical model compound of saxitoxin, phenylcarbamate. The recovery of this reaction was circa 80%. Since this recovery was achieved with a model compound instead of the relevant toxin, saxitoxin, it was decided not to further optimize the conditions of this reaction. The protocol for the immobilization of saxitoxin was prepared, based on the experience with the model compound. This reaction will be performed in the near future and results will be reported in the final report (Therapy after exposure to toxins (II)).

4.6 In summary

Preliminary results show that it is feasible to proceed with the development of aptamers as therapeutic tools. In case of exposure of military personnel to military relevant toxins, a rapid start of an efficient therapy may contribute to reduced mortality, morbidity and hospital costs. Ultimately, this will result in a better management of diseased military personnel.
It is anticipated that the optimized PCR method, together with the second, larger DNA library and the corresponding primers can also be used for the development of other aptamers as well.
The SELEX protocol that will be followed in future experiments is shown in Figure 10. In this protocol, both positive selection and negative selection will be incorporated. Based on the results with a chemical, nontoxic model compound of STX, it is anticipated that STX can be immobilized on magnetic beads (Mb) from CPG. This complex will be mixed with a 5-fold excess of DNA library during 1 h at room temperature (37 °C in some rounds). Bound DNA will be removed from the magnetic beads at 96 °C after the addition of water, followed by amplification with PCR. Product will be separated with 10% PAGE (or Low Range Agarose gel, 3%) and purified with a Gel extraction kit. The biotinylated (b) PCR product will be separated into ssDNA by means of streptavidin beads (SA-Mb). The ssDNA that was originally not coupled to biotin, which is an exact copy of the original bound DNA, will be amplified by PCR. This amplified product will be added to the immobilized STX in a subsequent round of SELEX.

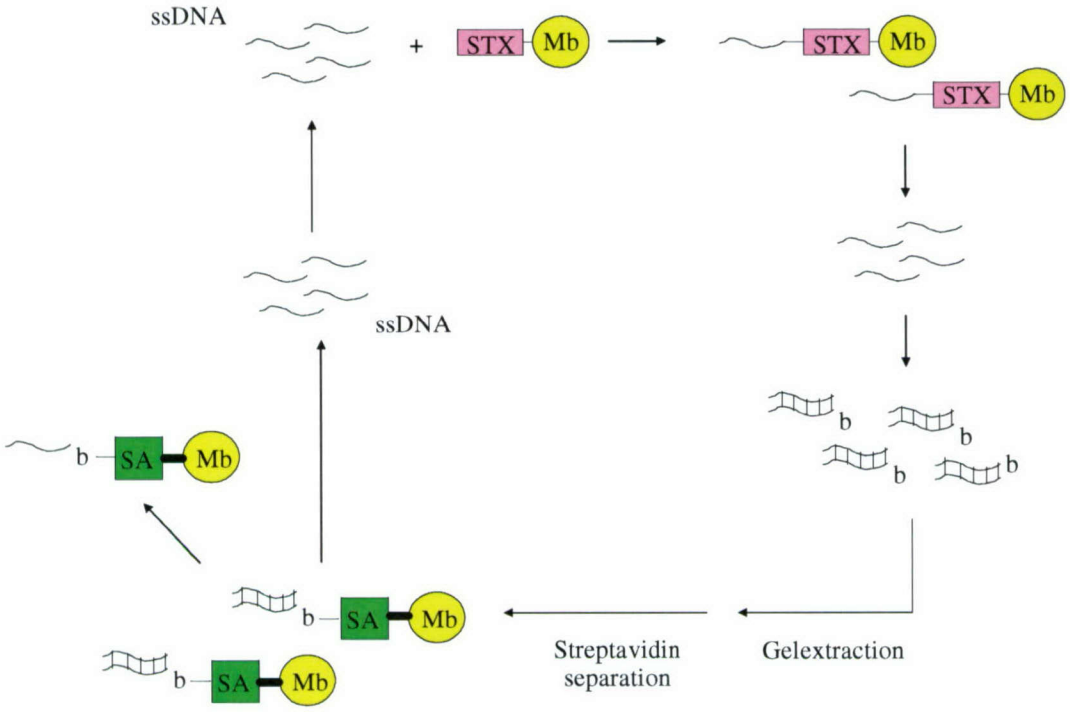


Figure 10 Saxitoxin (STX), immobilized on magnetic beads (Mb) from CPG will be mixed with a 5-fold excess of DNA library during 1 h at room temperature (37 °C in some rounds). Bound DNA will be removed from the magnetic beads at 96 °C after the addition of water, followed by amplification with PCR. Product will be separated with 10% PAGE (or Low Range Agarose gel, 3%) and purified with a Gel extraction kit. The biotinylated (b) PCR product was separated into ssDNA by means of streptavidin beads (SA-Mb).

4.7 Problems to be addressed

Aptamers (oligonucleotides) are extremely sensitive to nuclease activity. As a consequence, the half life of such compounds will be very short when they are used as therapeutics. However, methods are available to modify the aptamers in such a manner that they can remain in the body over a longer period of time. Modification of the aptamer can also be performed in order to create a higher affinity against the target molecule. But, it must be tested whether such modifications can be achieved without loss of bioactivity of the aptamer.

4.8 Applications in other areas of research

Aptamers can be used in other areas of research as well. For detection and identification, aptamers can be used in sensors. For diagnostic purposes and dosimetry, aptamers against specific biomarkers of exposure can be coupled to, for instance, magnetic beads to isolate specific biomarkers from the biological matrix. Aptamers can also be used to decontaminate both man and equipment.
Aptamers can be developed against peptide and non-peptide compounds, including the TIC's (toxic industrial chemicals).

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6 Signature

Rijswijk, September 2005

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15. ABSTRACT (MAXIMUM 200 WORDS (1044 BYTE))

During their duties, military personnel can be exposed to military relevant toxins. Then, efficient therapy is needed. Alternative antibodies, aptamers, can be used to neutralise and remove the toxins from the body. Aptamers are developed by means of the SELEX technology (Systematic Evolution of Ligands by EXponential enrichment). The results obtained in this report suggest that it is worthwhile to further develop aptamers against the neurotoxin saxitoxin. Development of aptamers that can be used as therapeutic agents can be administered to military personnel as antidotes after exposure has occurred. The development of an efficient therapy will contribute to fulfilling one of the main objectives of the Ministry of Defence in the area of 'Passive NBC Protection', i.e. 'Protection of personnel against adverse health effects as a result of (non intentional) exposure to biological agents under operational circumstances'. In addition, it is anticipated that the optimized PCR method, together with the developed DNA library and the corresponding primers can also be used for the development of aptamers against other targets as well.

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