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13. ABSTRACT (Maximum 200 words)

The objective was to develop novel thioaptamers targeting NF-□appaB/Rel proteins and other relevant proteins, allowing one to modulate the immune response to a BW attack. Combinatorial selection and structure-based design methods were used to develop thioaptamers to bind key proteins in response to pathogens such as hemorrhagic fever viruses and West Nile encephalitis virus. Two lead thioaptamers have been developed and tested against several viral animal models. XBY-6 bound p50 homo- or heterodimer of NF-□appaB, and XBY-S2 bound AP-1 transcription factors. XBY-6 in liposomes prolonged the incubation period of guinea pigs infected with P18 (virulent) Pichinde arenavirus, however by 3 weeks nearly all animals were dead despite the treatment while XBY-S2 protected up to 80% of the animals from death. XBY-6 and XBY-S2 also both protected mice that were later infected with West Nile virus. Other thioaptamers were selected from *in vitro* aptamer libraries to bind additional NF-□appaB dimers, TGF-β and various flavivirus envelope protein domain III proteins. In addition libraries of thioaptamers were synthesized on beads, one thioaptamer-one bead as well as library of libraries. The beads, when reacted with specific proteins, and the thioaptamers binding specific proteins were selected by flow cytometry.

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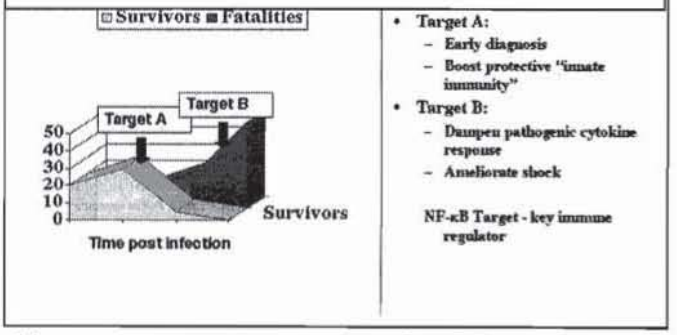
DAAD19-01-1-0379 Final Progress Report (3/7/01-5/31/05)
David G. Gorenstein, PI, UTMB

Statement of the Problem Arenavirus hemorrhagic fevers are fulminant human diseases of potential bioterrorism concern. Hemorrhagic fever viruses (category A bioweapon agents) have reportedly been weaponized by the former Soviet Union and the United States. Apart from supportive therapy, the only drug for treating arenavirus infections is ribavirin and it is only partially effective. Obviously, there continues to be an urgent need to expand the current therapeutic armamentarium. The pathogenic mechanism of arenaviruses is believed to involve dysregulation of cytokines and failure of innate immune mechanisms. Transcription factors of the NF-κB and AP-1 families are intimately involved in the regulation of a variety of immune and inflammatory responses. With DARPA funding we have developed new thioaptamer-based therapeutics to modulate pathogen-induced inflammatory responses. In particular we have identified key transcription factors (e.g., NF-κB and AP-1) challenged with selected biothreat (BT) agents. We have developed several decoy phosphorodithioate oligodeoxynucleotides (S_2-ODN) representing binding sites for specific transcription factors that can compete for protein binding to the authentic binding elements in cellular DNA. S_2-ODN are excellent candidates for aptamer or decoy strategies for modifying cell signaling and transcription. Additional advantages of thioaptamers include high affinity and specificity for protein targets, indefinite shelf life, low cost of production, and high reproducibility in quality control. We hypothesized and demonstrated that appropriate modulation of transcription factor activity within target cells of an arenavirus as well as flavivirus infected host using decoy S_2-ODNs ameliorate disease manifestations. Several first-generation thioaptamer leads have been identified and shown in preliminary animal therapeutic dosing to increase survival in animal models of infection with the flavivirus West Nile and the arenavirus Pichinde. We have also developed novel combinatorial methods to optimize these lead thioaptamers and develop second-generation phosphorothioate (S-ODN) and phosphorodithioate (S_2-ODN) oligonucleotide "thioaptamers" targeted towards transcription factors, AP-1 and NF-κB. Specific initial arenaviral targets include Pichinde, Lassa and Junin viruses (the latter on both the NIH and CDC class A lists).

Our goal over the next several years (post-DARPA) is to complete an IND for an optimal therapeutic thioaptamer(s) for Lassa and Junin arenaviruses.

Summary of Results

Figure 1. Schematic representation for immune responses post infection. Target A represents immune response clearing virus with patient survival. Target B represents cytopathogenic immune response resulting in shock.



Viral induced changes of cellular transcription factors such as RBP-Jκ and NF-κB that we first described (**Fennewald *et al.*, 2002; references in bold, supported by DARPA**) as well as changes in CREB, AP-1 and interferon (IFN) response factors (unpublished results) are likely to contribute to pathogenesis and the failure of IFN to control the virus. Available data thus suggest that suppression of pro-inflammatory responses early in infection allows for unchecked viral replication, and leads to secondary upregulation of pro-inflammatory cytokines late in infection. Therefore, manipulation of cytokine responses could be beneficial at two levels; early boosting of protective inflammatory responses (target A) and/or late suppression of pathogenic systemic inflammatory response (target B, Figure 1).

Our collaborative DARPA team has been working to develop thioaptamer countermeasures against BT agents including arenaviruses and flaviviruses.

ODN Agents: thioaptamers RNA and DNA oligonucleotides (ODNs) can act as "aptamers," (i.e., as direct *in vivo* inhibitors selected from combinatorial libraries) for a number of proteins and transcription factors such as human NF-κB (**King et al., 2002; Bassett et al., 2002**). Among a large variety of modifications, S-ODN and S_2-ODN render the agents more nuclease resistant. The first antisense therapeutic drug uses a modified S-ODN (CIBA Vision, A Novartis Company). The S_2-ODNs also show significant promise, however, the effect of substitution of more nuclease-resistant thiophosphates cannot be predicted, since the sulfur substitution can lead to significantly decreased (or increased) binding to a specific protein (**King et al., 2002**) as well as structural perturbations (**Volk et al., 2002**) and thus it is not possible to

predict the effect of backbone substitution on a combinatorially selected thioaptamer. Thus, if at all possible, selection should be carried out simultaneously for phosphate ester backbone substitution as well as the base sequence.

Phosphorodithioate analogues Gorenstein's and Caruthers' laboratories independently synthesized an important class of sulfur-containing oligonucleotides, the dithiophosphate S_2-ODNs. In fact, ours was the first patent awarded for this class of agent (**U.S. Patent 5,218,088, 1993**; see also **Gorenstein, et al., U.S. Patent, #6,423,493, 2002 and Patents Pending, 2002, 2003a,b,c,d)** The dithioates contain an internucleotide phosphodiester group with sulfur substituted for both nonlinking phosphoryl oxygens, so they are both isosteric and isopolar with the normal phosphodiester link, and are also highly nuclease resistant.

Thiophosphate thioaptamers binding to proteins Importantly, it has been noted that sulfur substitutions of the phosphoryl oxygens of oligonucleotides often leads to their enhanced binding to numerous proteins **(Yang et al., 2002; King et al. 2002**). Oligonucleotides with high monothio- or dithiophosphate backbone substitutions thus appear to be "stickier" towards proteins than normal phosphate esters, an effect often attributed to "non-specific interactions." However, as we have suggested, one explanation for the higher affinity of the thiosubstituted DNAs is the poor cation coordination of the polyanionic backbone **(Volk et al., 2002).**

Even in specific protein-nucleic acid contacts, sulfurization of the internucleotide linkages can lead to enhanced binding (**King et al. 2002**). However, the "thioate-effect" can also lead to decreased binding as well, and it is not possible to predict whether there will be an increase or decrease in binding.) We can take advantage of this "stickiness" to enhance the specificity and affinity of thio- and dithiophosphate agents for a protein target. However, we need to optimize the total number of thioated phosphates to decrease non-specific binding to non-target proteins, and enhance only the specific favorable interactions with the target protein. With DARPA funding we have developed two technologies for selecting these thioaptamers.

In Vitro Combinatorial Selection of Thiophosphate Aptamers A recent advance in combinatorial chemistry has been the ability to construct and screen large random sequence nucleic acid libraries for affinity to proteins or other targets. The aptamer nucleic acid libraries are usually selected by incubating the target (protein, nucleic acid or small molecule) with the library and then separating the non-binding species from the bound. The bound fractions are then amplified using the polymerase chain reaction (PCR) and subsequently reincubated with the target in a second round of screening. These iterations are repeated until the library is enhanced for sequences with high affinity for the target. However, agents selected from combinatorial RNA and DNA libraries have previously always had normal phosphate ester backbones, and so would generally be unsuitable as drugs or diagnostics agents that are exposed to serum or cell supernatants because of their nuclease susceptibility. The effect of substitution of nuclease-resistant thiophosphates cannot be predicted, since the sulfur substitution can lead to significantly decreased (or increased) binding to a specific protein.

In our recent publications (**King et al., 2002; Yang et al., 2002a,b, 2003**), and patent awards and applications (**Gorenstein, et al., U.S. Patent, #6,423,493, 2002 #6,867,289, 2005, and Patents Pending, 2002, 2003a,b,c,d)** we described the combinatorial selection of phosphorothioate oligonucleotide aptamers from random or high-sequence-diversity libraries, based on tight binding to the target (e.g. a protein or nucleic acid) of interest. A hybrid monothiophosphate backbone combinatorial library was created by PCR, using an appropriate dNTP(αS) in the Taq polymerization step. We have demonstrated that combinatorial thiophosphate duplex and single-stranded (ss) libraries can be successfully screened for binding to a number of different protein and nucleic acid targets, including NF-IL6, NF-κB, HIV reverse transcriptase, Venezuelan Equine Encephalitis nucleocapsid (using an RNA thioaptamer), HepC IRES nucleic acid, Flavivirus envelope protein domain III, TGF-β and others, including a protein involved in CpG-induced "innate immunity".

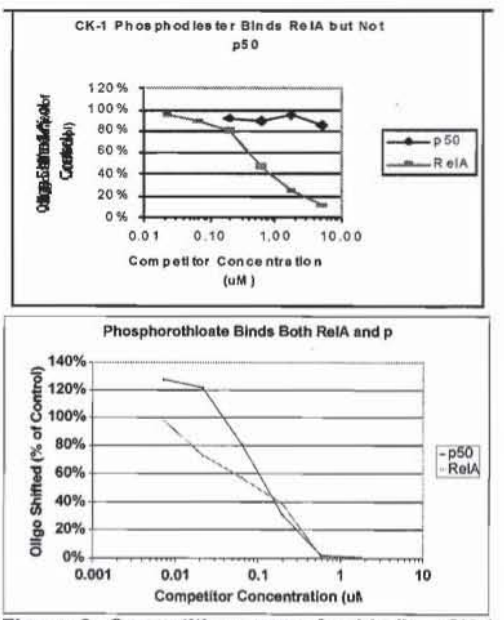


Figure 2. Competition assay for binding CK-1 specific for NF-κB 42-mer aptamers. In standard competitive binding assays, ^{32}P -IgkB promoter element ODN duplex was incubated with recombinant p50 or p65 and competitor oligonucleotide (Left: phosphodiester CK-1; Right: phosphorothioate CK-1). The reactions were then run on a nondenaturing polyacrylamide gel, and the amount of radioactivity bound to protein and shifted in the gel was quantitated by direct counting.

NF-κB thioaptamers We performed both *in vitro* thioselection and rational design of thioaptamers against NF-κB (**Gorenstein, et al., U.S. Patent, #6,423,493, 2002, #6,867,289, 2005, and Patents Pending, 2001, 2002, 2003a,b,c,d; King et al., 2002; Bassett et al., 2004**). Sharma and co-workers at Roche, Inc. previously demonstrated effective aptamer inhibition of NF-κB activity. They further achieved inhibition of NF-κB in cell culture using S-ODN duplex decoys with an NF-κB binding consensus-like sequence (dGGGGACTTCC). Our initial approach used the "CK-1" 42-mer duplex oligonucleotide identified by Sharma *et al.* The wild-type CK-1 duplex sequence contains 3 tandem repeats of a 14-mer NF-κB consensus-like sequence (5'-CCA GGA GAT TCC ACC CAG GAG ATT CCA CCC AGG AGA TTC CAC 3').

S-ODN CK-1 Monothioate Aptamers. As pointed out earlier, it is unlikely that the phosphodiester form is appropriate for therapeutics or diagnostics because of its short half-life in cells, cell extracts and serum. The phosphorothioate and dithioate internucleoside modifications are therefore needed for *in vivo* studies of such compounds. Our studies, using recombinant protein homodimers of p50, p65, and c-Rel, confirmed published results showing that the CK-1 sequence could bind to and compete for binding to p65 homodimer, but not p50/p50, in standard electrophoretic mobility shift assays (EMSA) [Figure 2 (top)]. However, in contrast, the fully substituted phosphorothioate CK-1 aptamer [Figure 2 (bottom)] **inhibited p65/p65 and p50/p50 equally**. This again confirms that S-ODNs with many phosphorothioate linkages are "sticky" and tend to bind proteins non-specifically. We have found that if the number of phosphorothioate linkages is decreased to only 2-6, specificity can be restored, but binding is not enhanced. **The original publications by the Roche company authors described only the specificity of the phosphodiester oligonucleotides, and did not address the problem of altered specificity of the phosphorothioates.**

Table 1. Comparison of sequences from the various NF-κB dimer selections.

Selection	Sequence
p65/p65	*CGGGGTGTTGTCCTGTGC TCC*
p50/p50	*GGGGTTCCACCTTCACTGGGCG*
p65/p50	*TGGGGCTGTACAGGGTGC AC*
p65/p65-edited, or	

We attempted to repeat the binding inhibition studies using cell extracts, we encountered unexpected difficulties. The diester form of the CK-1 aptamer did not compete effectively for NF-κB binding in cell extracts derived from two different cell lines: the 70Z pre-B cell line and the RAW 264.7 mouse macrophage-like line. The heterodimers in these cells either do not bind the CK-1 sequence tightly enough, or it is bound by other cellular components. **Therefore, even sequences with good binding and specificity in the diester form, when fully thiophosphate-substituted, lose their sequence specificity.** Thus, this stickiness makes the characterization of fully thioated aptamers *in vitro* not necessarily predictive of their activities *in vivo*.

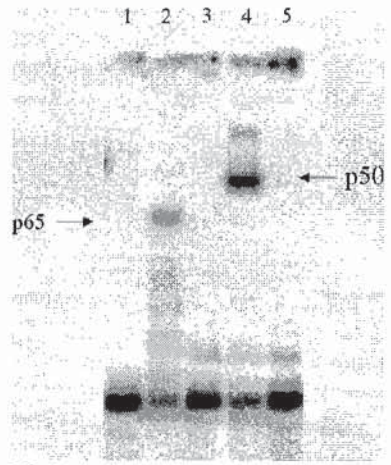


Figure 3. Figure 4. NF-κB subunit binding to Radiolabeled XBY6 dithioate. ODN aptamer was incubated with 70Z/3 cell nuclear extract in the presence (lanes 2 and 5) or absence (lanes 1,3,4) of anti-p50 antibody. Protein-bound ODN duplex was separated on a standard gel. Lanes 1 and 2: ^{32}P -IgkB promoter element. Lane 3: ^{32}P -phosphodiester CK1. Lanes 4 and 5: ^{32}P -XBY6 oligonucleotide.

Thioselection against NF-κB (p50:p50, p65:p65) As described in **King et al. (2002)** a unique thiophosphate duplex library was screened for binding to the p50 homodimer. A thioaptamer representing this sequence was generated by PCR amplification using a biotinylated reverse primer. Binding studies were initiated using a chemiluminescent EMSA. This biotinylated thioaptamer binds tightly to p50; the sequences are different from those obtained for *in vitro* combinatorial selection against p65 homodimers (**King et al., 2002).** The chemically synthesized phosphorothioate aptamers are a diastereomeric mixture of both Rp and Sp configurations. The thioaptamers bind and compete for the same NF-κB site as the known promoter element IgkB (K_d = 78.9 ± 1.9 nM for a Rel A-selected thioaptamer, and 19.6 ± 1.25 nM for a p50-selected thioaptamer). The normal phosphate ester backbone version of the Rel A selected aptamer binds Rel A with a K_d of 249.1 ± 1.8 nM. The p50 dimer-selected chiral thioaptamer binds to p50 with affinities below 5 nM under conditions where no binding to p65 is observed. Similarly, the p65 dimer-selected chiral thioaptamer binds to p65 dimers with affinities below 5 nM under conditions where no binding to p50 is observed. These EMSA binding experiments demonstrate that the enhanced affinity can be attributed to the presence of sulfur. Collectively, these results further demonstrate the feasibility of the thioaptamer selection technology as a method for producing specific, high-affinity ligands to proteins. We have also demonstrated that the chemically synthesized (mixed diasteromer) thioaptamers bind tightly in cell

nuclear extracts to both the p50:p65 heterodimer and p50:p50 homodimer. However, the enzymatically synthesized, chiral thioaptamer selected against the p50 homodimer **only binds to p50:p50 in nuclear extracts (Fig. 3; King et al., 2002; Gorenstein, et al., U.S. Patent, #6,423,493, 2002)**.

Cytokines and Arenavirus hemorrhagic fevers: One rationale for targeting transcription factors such as AP-1 and NF-κB in arenavirus hemorrhagic fevers has to do with the putative importance of cytokine responses in disease or protection from disease. Pro-inflammatory cytokines have cardiovascular effects such as endothelial activation, increased vascular permeability, pro-coagulant effects, and myocardial suppression, which are features of the terminal pathophysiology of hemorrhagic fevers. Soluble mediators of systemic inflammation, such as cytokines, have long been suspected as important causative factors in the fulminant clinical course of arenavirus hemorrhagic fevers. However, observations are incomplete and somewhat conflicting concerning the potential roles of these cytokines in arenavirus disease. Elevated serum TNF-like bioactivity was associated with virulence in PIC-infection of guinea pigs **(Aronson, Herzog et al., 1995)**. Human clinical studies of Argentine hemorrhagic fever patients suggested that elevated levels of TNF-α and IL-8 correlate with clinical severity. Similarly, our research found that PIC suppresses production of TNF-α and IL-6 in a mouse macrophage cell line, while failing to produce the NF-κB and RBP-Jκ transcription factor alterations associated with macrophage activation **(Fennewald, Aronson et al., 2002)**.

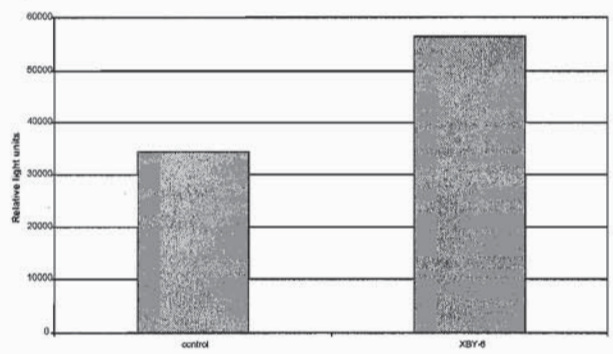


Figure 4 Effect of XBY-6 on NF-kB dependent reporter gene expression

Over the past several years with DARPA funding, we have been testing two lead candidate thioaptamers (XBY-6 and XBY-S2 identified in an earlier DARPA-funded project) in the guinea pig PIC model of Lassa fever. In this project, NF-κB and AP-1 were being targeted because of the key role of these families of transcription factors in regulation of a variety of genes involved in inflammation and immunity. Studies by Herzog and others, have shown that attenuated (P2) and virulent (P18) variants of PIC induce strikingly different patterns of NF-κB activation, both in cultured macrophage cell line (P388D1), and in macrophages explanted from peritoneal cavities of PIC-infected guinea pigs **(Fennewald et al, 2002)**. This data suggested that P2 infection induces an "activated macrophage" pattern of NF-κB and RBP-Jκ transcription factors, whereas P18-infected macrophages resembled quiescent, or mock-infected cells. We reasoned that, since the activated P2 pattern was associated with attenuated disease, that treatment of macrophages that restored this response could ameliorate disease caused by the virulent P18. XBY-6 is a phosphorothioate oligonucleotide 14-mer, with phosphoryl oxygens replaced by sulfur groups at 6 locations. XBY-6 binds p50 homodimers in nuclear extracts of cultured cells. Preliminary animal testing strategies were based on this evidence that XBY-6 serves as a potential decoy for p50 homodimers, which are believed to be repressors of transcription. Indeed, using a reporter cell line we developed, in which 293T cells are stably transfected with reporter plasmid containing luciferase gene with an NF-κB dependent enhancer element, XBY-6 increased reporter gene activity as expected (Fig 4). Cell culture experiments using macrophage cell lines (P388D1), and primary guinea pig macrophages, also showed that XBY-6 pre-treatment of cells boosted TNF and IL-8 responses.

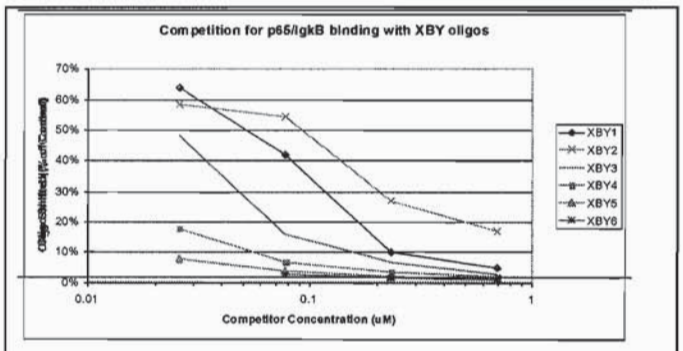


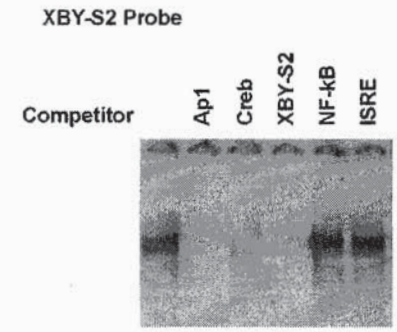
Figure 5. Inhibition of p65 homodimer binding by S_2-ODN (XBY's). In standard competitive bindingassays, the ^{32}P-IgkB promoter element ODN was incubated with recombinant p65 and varying amounts of XBY decoy competitor. The relative binding ability of the unlabeled ODNs was determined by the concentration needed to compete effectively with the standard labeled ODN. XBY1 through 6 correspond to CK-14 aptamers with 1-6 dithiophosphate substitutions, respectively **(Yang et al., 1999)**.

Dithiophosphate Aptamers Binding to Proteins

S_2-ODN CK-14 Dithioate Aptamers We also synthesized the CK-14 14-mer duplex with some randomly placed **dithioate** linkages (both of the non-bridging oxygens are replaced by sulfurs). These have proved very significant, as they altered binding specificity, and lack the extreme "stickiness" of the fully thioated aptamer. We tested several dozens of different S_2-ODN's CK-14 duplexes and found that with an increasing number of dithioate substitutions in the identical sequence, binding by the S_2-ODN increases dramatically (Fig 5). One of the tightest-binding dithioaptamers (XBY-6) contains 6 dithioate linkages on the two strands.

Significantly, the XBY-6 aptamer also binds to a single NF-κB dimer in cell extracts (Figure 3), while the **standard phosphodiester ODN shows no NF-κB-specific binding in extracts. Thus, we have succeeded in identifying by a random selection process a thioate backbone modification, which for the first time increases the specific binding of the oligonucleotide to NF-κB above that to other cellular proteins (Yang and Gorenstein, 2004 and earlier papers referenced therein; Gorenstein, et al., U.S. Patent, #6,423,493, 2002).** As shown in Figure 3, our lead XBY-6 shifts one complex in nuclear extracts from 70Z/3 cells. By using specific antibodies to supershift the complex, we have identified p50 as one component of the complex. Since XBY-6 binds more tightly to p50/p50 than p65/p65, the shifted band is likely to represent the p50 homodimer. This band does not co-migrate with either the p50/p50 or p50/p65 bands, but the change in the altered chemical structure changes the mobility of the ODN. Only one major band is seen, however, even though the lysate contains at least two major distinguishable NF-κB complexes (p50 homodimers and p50/p65 heterodimers).

These data demonstrate the feasibility of altering the binding specificity and affinity by substituting only a limited number of internucleoside linkages. We also appear to have an aptamer which can distinguish among various NF-κB dimers within the cell. We can, therefore, use this thioaptamer to bind to **a single NF-κB dimer within cell supernatants and even inactivate target dimers within whole cells and animals**. We have also found that when guinea pigs were injected with XBY-6 and lipopolysacccaride (LPS) to induce inflammatory response, we observed an increase in the levels of TNF-α above that seen after injection with LPS alone. In animal macrophage extract studies, we found that **XBY-6 eliminated a single p50 (or p105) dimer band** on EMSAs. Since the p50 homodimer is believed to be a transcriptional inhibitor of the immune response, these data demonstrate the ability to target a single protein within live animals, and the feasibility of altering the binding specificity by substituting only a limited number of internucleoside linkages **(Gorenstein et al. patents pending, 2002)**.



P388D1 macrophage cells polyIC induced

Figure 6–XBYS2 aptamer specifically binding to proteins in P388D1 cell nuclear extracts

We have been able to demonstrate the 1:1 binding stoichiometry of p65 to the 22mer binding site known as IgκB with a K_d near 4 nM. Our best dithiophosphate aptamer, XBY-6, has a binding affinity to p65 homodimer of 1.4 nM and **sub-nM to p50 (King et al., 2002)**.

XBY-S2:
5'-CC AGT$_{S2}$G ACT$_{S2}$C AGT$_{S2}$G-3'
3'-GG$_{S2}$TCA C$_{S2}$TGA G$_{S2}$TCA C-3'

AP-1 Thioaptamer

The AP-1 family also plays a key role in the immune response and represents another target. The AP-1 family also plays a key role in the immune response and may represent a target in the treatment for infectious agents. We have also been able to identify another thioaptamer (XBY-S2) with six dithioates and an AP-1-type consensus sequence which bound to a subset of AP-1proteins (in preparation). Our lead thioaptamer XBY-S2 was found to specifically bind AP-1 proteins from a macrophage like cell line (P388D1) (Fig. 6) and also human recombinant c-jun AP-1 dimers (data not shown). To determine the full spectrum of active AP-1 proteins present in PolyI/C stimulated P388D1 cells, we used specific antibodies and the consensus AP-1 binding site (top panel). P388D1 cells have AP-1 complexes including Fra2, c-Fos, JunB, JunD and c-Jun. We were unable to detect Fra1, ATF2, and CREB. We performed the same experiment to identify the AP-1 proteins that bind to XBY-S2. We observed that c-Fos, Fra2, JunB and possibly c-Jun bound XBY-S2. Therefore, it appears that a subset of AP-1 proteins present in P388D1 cells recognize the XBY-S2 thioaptamer. (Fig 7; **Gorenstein, et al., Patents Pending, 2003a,b,c,d**).

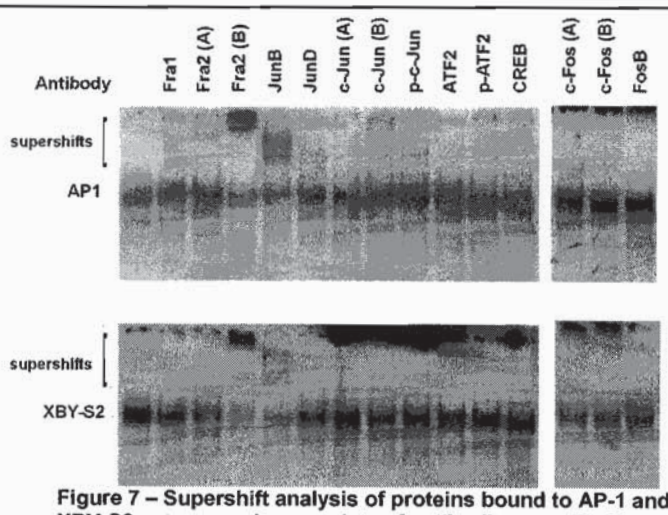


Figure 7 – Supershift analysis of proteins bound to AP-1 and XBY-S2 aptamer using a variety of antibodies specific for various members of the AP-1 transcription factor family.

Treatment of P388D1 cells with XBY-S2 eliminates AP-1 DNA binding activity. XBY-S2 includes a consensus AP-1 binding site (5'-TGAG/CTCA-3') and when used as a decoy aptamer, it would be presumed to influence the expression of cytokines from macrophages by sequestering AP-1 proteins. In order to establish that XBY-S2 functions as a decoy, P388D1 macrophage cultures were

treated with liposomes with and without the indicated aptamers for 24 hours prior to stimulation with poly I/C. The XBY-6 is a thioaptamer that contains the same number of adenine and guanine residues as well as the same number (6) of dithioate substitutions but targets NFκB p50/p50 and serves as an ideal negative control. Nuclear extracts were analyzed by EMSA with the AP-1 and RBP-Jκ oligonucleotide probes. Treatment of cells with XBY-S2 completely eliminated the AP-1 binding present in polyI/C stimulated macrophages (Fig. 8). XBY-S2 elimination of AP-1 DNA binding activity was specific in that it did not eliminate RBP-Jκ DNA binding activity. Therefore, the XBY-S2 thioaptamer appears to be an efficient inhibitor of AP-1 transcription factor DNA binding.

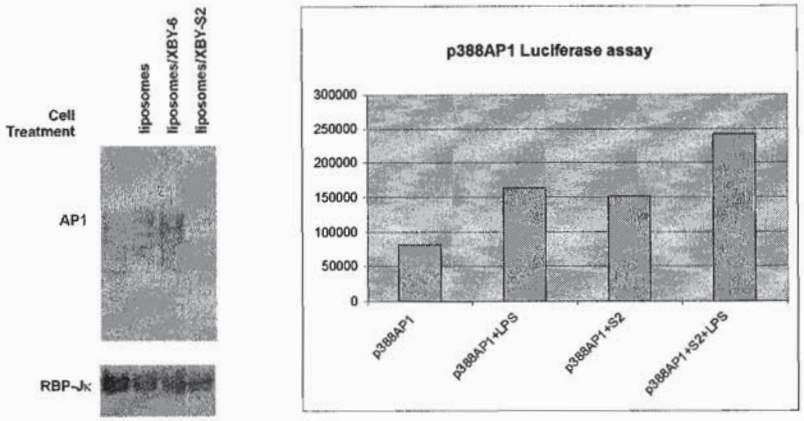
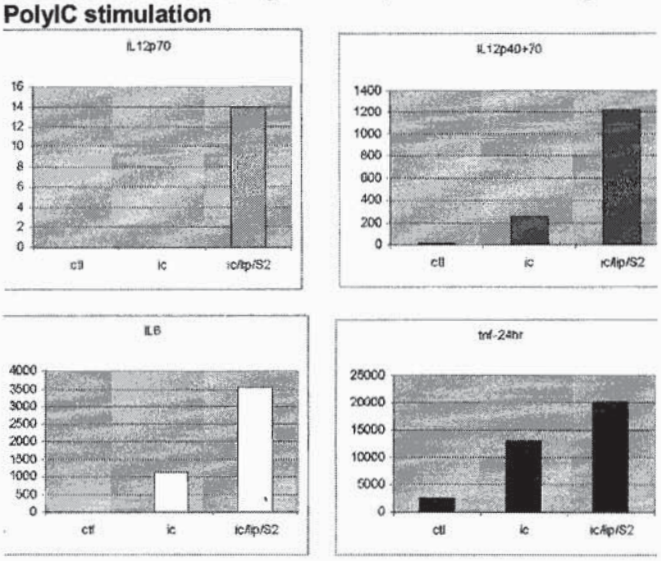


Figure 8 –XBY-S2 eliminates AP1 DNA binding activities in macrophages and alters AP-1 reporter gene expression in P388D1 cells. Panel A. P388D1 cells were incubated with liposomes with or without the indicated thioaptamers for 24 h prior to stimulation with poly I/C. Nuclear extracts were analyzed by EMSA with the AP-1 and RBP-Jκ oligonucleotide probes. Panel B. P388D1 cells stably transfected (P388AP1) with an AP-1 drive luciferase reporter plasmid, pHTS-AP1 were stimulated with 100ng/ml of LPS for 6 hours with and without pretreatment overnight with 10μg/well XBY-S2 and then assayed for luciferase activity.

Reporter assays confirm that XBY-S2 treatment of P388cells inhibits AP-1 transcription factor activity. In order to confirm that the XBY-S2 thioaptamer altered AP-1 regulated transcription, we established a P388D1 derived cell line (P388AP1) stably transformed with an AP-1 driven luciferase reporter plasmid, pHTS-AP1 (Biomyz, San Diego, CA) that contains a tandem repeat of 6 copies of an AP-1 site (TGACTAA) linked to the luciferase gene. In Fig. 8, P388AP1 reporter cell lines respond to LPS stimulation with a nearly 3-fold increase in luciferase activity. Treatment with XBY-S2 alone stimulates nearly the same increase in luciferase expression as LPS stimulation. LPS plus XBY-S2 results in an additive increase the level of luciferase expression. Treatment with a thioaptamer without an AP-1 site has no effect on AP-1 (data not shown). Therefore, it appears that XBY-S2 is acting to block the activities of AP-1 proteins that repress transcription from an AP-1 driven promoter.

Figure 9- Pretreatment of P388D1 cells with XBY-S2 increases the level of cytokine expression following PolyIC stimulation



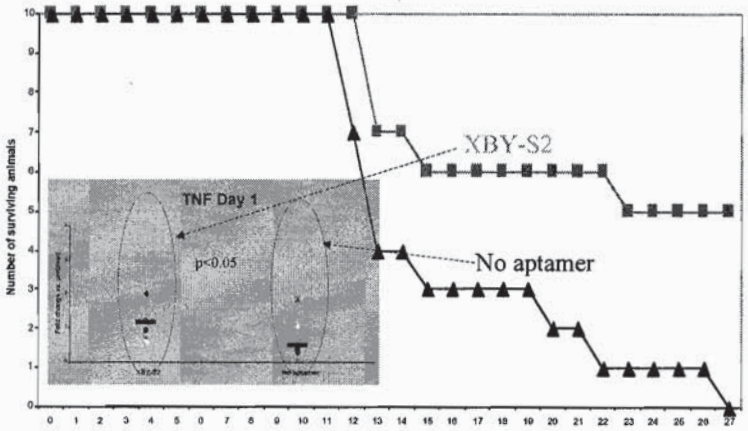
XBY-S2 perturbation of interleukin expression in P388D1 cells. PolyI/C is a potent inducer of macrophage cytokine expression and can be used to mimic the activation of cells by viral ds RNA. To determine if XBY-S2 can influence cytokine gene expression, we measured the expression of cytokines elaborated from macrophages with and without stimulation with poly I/C with and without prior treatment with XBY-S2. In Fig. 9, stimulation with poly I/C with XBY-S2 increases the expression of IL12 (6-fold), IL6 (3.5-fold), TNFα (1.7-fold) over the levels expressed in cells stimulated with PolyI/C alone. These data suggest that XBY-S2 modulates AP-1 proteins thereby altering the expression of proinflammatory cytokines that could influence innate immunity induced in response to viral challenge. These data also suggest that either AP-1 proteins binding to the XBY-S2 decoy aptamer repress the transcription of these cytokines, or AP-1 regulates the expression of another protein that serves as a repressor. XBY-S2 also induced a dose-dependent enhancement of cytokine basal but not LPS-induced IL8 and TNF-α secretion from primary guinea pig peritoneal macrophages (data not shown).

In two separate experiments, liposomal (Tfx50 from Promega) thioaptamer was administered intraperitoneally to 400 g male guinea pigs at day 0 and 2 of infection with virulent PIC P18. Data from a representative experiment (Fig. 10) revealed a statistically significant increased survival in XBY-S2 treated animals compared to animals that received virus only. During this experiment, blood was sampled at serial time points for all animals, and cytokine mRNA levels were

quantitated by realtime RT-PCR. Results are expressed as fold change of cytokine mRNA compared to prebleed levels for that same animal. Among the cytokines tested, two showed statistically significant differences between XBY-S2 and no aptamer groups at day 1 post- infection; there was a small but statistically significant elevation of TNF-α mRNA in treated compared to untreated groups, and a significant depression of the chemokine GRO in treated compared to untreated animals (Fig. 10 insert). These results support the idea that these thioaptamers may boost early beneficial cytokine responses. It is likely that better protection could be achieved with different dosing strategies and alternate liposome formulations.

It is plausible that manipulation of NF-κB or AP-1 responses could interdict the pathogenetic sequence in arenavirus hemorrhagic fevers, or boost early protective innate immune responses. Indeed both XBY-6 and XBY-S2 show efficacy as first-generation therapeutics for a flavivirus, West Nile (where we observe 8/10 animal survival by treatment with XBY-S2, 5/10 animal survival with XBY-6 and 0/10 animal survival with 10 LD50 of WNV; data not shown). As shown above, these lead thioaptamers modulate a number of key cytokines (including TNF-α and others not shown) and **we hypothesize that appropriate modulation of transcription factor activity within target cells of an arenavirus infected host using decoy thioaptamers will ameliorate disease manifestations.**

Fig. 10: Effect of XBY-S2 on survival of P18 infected GP.



Guinea pigs received XBY-S2 in Tfx-50 liposomes on days 0 and 2 of infection with 1000 pfu of P18. Proportions of surviving animals are significantly different in the two groups (p<0.05, Fisher exact test). Inset indicates TNFa mRNA levels in blood cells sampled on day 1. Bar is mean for each group.

As shown in Figure 1, the ultimate goal is develop therapeutics in which specific thioaptamers will be used to modulate the immune response to BW agents – either to enhance the innate response (Target A) or to decrease the pathogenic response such as shock (Target B). The lead compound (XBY-S2) identified thus far targets the transcription factor AP-1, providing protection in animal models of West Nile and Pichinde virus suggesting that this compound may exhibit a broad anti-viral activity by some unknown mechanism. There are two possible mechanisms by which the AP-1 thioaptamer may be conferring protection: 1) XBY-S2 modulates the activities of cellular trancription factors that results in changes in the gene expression or 2) thioaptamers stimulate immunity through activation of Toll like receptors.

Significance Both NF-κB and AP-1 are important in the immune response and are affected by Pichinde virus and West Nile infection in the macrophage. As shown in Figure 10, we demonstrate that the survival of the Pichinde virus infected animals can be prolonged using our first-generation lead thioaptamers. *This data demonstrates that we can alter the outcome of in vivo viral infections by Category A agents by the manipulation of transcription factors.*

We now have two first-generation lead thioaptamers that can distinguish among various NF-κB and AP-1 dimers within the cell. One of the NF-κB decoys can **bind to a single NF-κB dimer in cell extracts or within a cell in either cell culture or animal studies**. The XBY-S2 thioaptamer also binds to a subset of possible AP-1 dimers in cells. These thioaptamers were identified by a laborious process of synthesizing a large number of potential drugs and screening for their binding or activity. As described above and in the next section, we have also developed various *in vitro* and bead-based HTS methods for thioaptamers which we believe will allow us to optimize the activity of our first-generation leads. These results point to the importance of our thiophosphate combinatorial selection methods to identify minimally substituted thioated oligonucleotides with high affinity, high binding specificity, and increased nuclease resistance *in vitro* and *in vivo*.

Phosphorodithioate and Phosphorothioate Aptamers via Split Synthesis Combinatorial Selection Identification of a specific aptamer based upon *in vitro* combinatorial selection methods is limited to substrates accepted by polymerases. One alternative to *in vitro* combinatorial selection methods would be one-bead one-compound based split synthesis which has been developed for bead-based selection of organic molecules, peptides and oligosaccharide libraries. Remarkably, the one-bead, one-aptamer split synthesis method had not been investigated for identifying a specific ODN aptamer that

targets proteins. We have recently developed the split and pool synthesis combinatorial chemistry method for creating a combinatorial library of thioated oligonucleotide agents (either phosphate, monothiophosphate, dithiophosphate or hybrid backbone (**Gorenstein et al, US Patents pending, 2002, 2003a,b,c,d; Yang et al., 2002b, 2003, 2004; Leary et al., 2005**). In this procedure each unique member of the combinatorial library is attached to a separate support bead. To introduce many copies of a single, chemically pure S-ODN thioaptamer onto each bead, we utilized a "mix and separate" split synthesis method utilizing a two-column DNA synthesizer. On completion of the automated synthesis, the column is removed from the synthesizer and deblocked. Importantly, with our coupling scheme using a non-cleavable hexaethyleneglycol linker attaching the first phosphoramidite (15 or 70 μm beads provided by ChemGenes), the thioaptamers are still covalently attached to the beads after complete deprotection. Each bead thus contains a single sequence with a specified backbone modification that is identified by the base (**Gorenstein et al, US Patents pending, 2002, 2003a,b,c,d; Yang et al., 2002b, 2003, 2004; Leary et al., 2005**). Targets that bind tightly to only a few of the 10^6-10^8 different support beads (each bead containing a unique thioaptamer) can be selected by binding the target protein to the beads and then identifying which beads have bound target by immunostaining techniques or direct staining of the target (see below). Our methodology allows us to **rapidly screen at rates of up to 10^9 beads/hr** from libraries of as many as 10^6-10^8 unique thioaptamers that bind to proteins such as NF-κB and AP-1 using a novel PCR-based identification tag of the selected bead (**Yang et al., 2002, 2003; patents submitted**).

For example, we applied this scheme to synthesize a library of $>10^6$ beads, each containing a unique thioaptamer. These libraries consisted of a 22-nucleotide "random" sequence (20 split/pool steps, or 2^{20} random thioaptamers) flanked by 11-15 nucleotide defined primer regions at the 5' and 3' ends (**Yang et al., 2002b, 2003**). A phosphorothioate linkage was introduced on every other base in column 2, following the "split and pool" approach. The single-stranded 52-mer S-ODN random library was converted to double-stranded DNA by Klenow DNA polymerase I (Promega) reaction in the presence of DNA polymerase buffer, dNTP mix and downstream primer. Therefore, the one strand of the duplex potentially contained S-ODN modifications and the other complementary strand was composed of ODN. A duplex DNA library in which both strands contain S-ODN modifications could also be generated using a Klenow reaction with no more than three dNTP(α)S.

Selection. We have also demonstrated the successful application of high throughput/multi-color flow cytometry and bead sorting to screen aptamer bead libraries for those beads which bind to, e.g., a target protein (**Yang et al., 2003; Leary, 2005; Gorenstein et al, US Patents pending, 2002, 2003a,b,c,d**). Modifications were made to a custom-built flow cytometer in James Leary's facility (UTMB, now Purdue) to make it more amenable to bead identification and isolation. For example, bead fluorescence and forward scatter were the two parameters chosen for real-time characterization of each aptamer bead passing the first sort point of the custom-built flow cytometer/sorter. In operation, "positive" beads (containing thioaptamer-bound target protein, fluorescently labelled with Alexa 488 dye) were easily sorted from negative beads. The flow-sorted "positive" beads can then be subjected to one-bead PCR to identify the thioaptamer that binds the target protein. As shown in Fig. 11, and as a demonstration of the use of the one-bead, one-ODN:protein system with dual color sorting (**Yang et al., 2003**), the IgκB dsDNA consensus sequences with a chemically synthesized 5'-amino-linker were immobilized onto 15-20 μm polystyrene microspheres. The DNA-bound beads were then incubated with purified p50 and p65 proteins, respectively. DNA transcription factor complexes were detected with primary Antibodies specific for the p50 and p65 proteins, followed by an additional incubation with Alexa 488-conjugated secondary Abs for p50 and PE-conjugated secondary antibody for p65. The beads were viewed by fluorescent microscopy and then analyzed on the UTMB MCU's HiReCS system. We have also screened XBY-6 beads against WI-38 VA13, an SV40 virus-transformed human fibroblastic cell line extract by direct fluorescence microscopy.

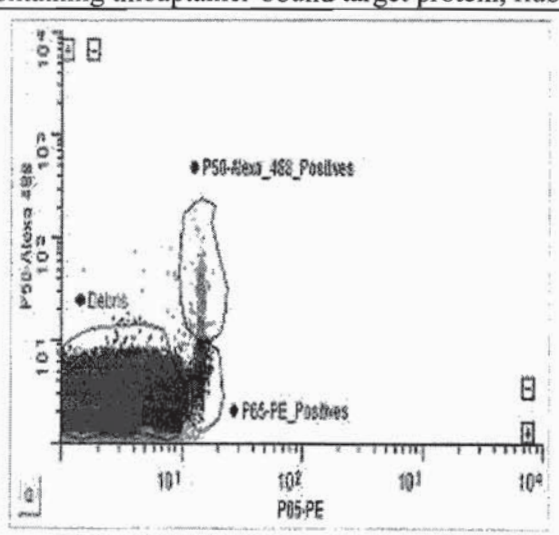


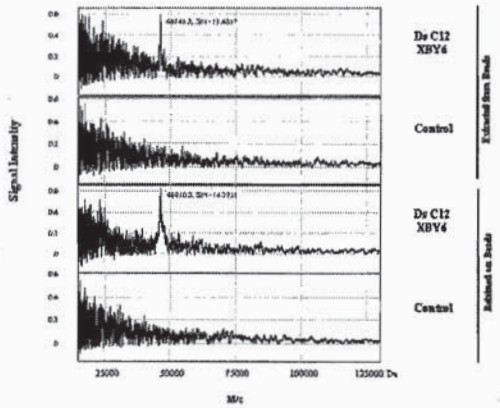
Figure 11 - Shows a mixed bead population. The majority of the "Debris" population are the 0.8 micron carrier beads that were used to bring up the volume of the samples,(as well as to model a very high number of negative beads) since the beads were at a very low dilution.

Sequencing on the bead Each individually selected bead was washed thoroughly with 8 M urea (pH 7.2) to remove the protein and was directly used for our "one-bead PCR" amplification using the 5' and 3' end primers (**Yang et al., 2003; Yand and Gorenstein, 2004; Leary et al., 2005**). The PCR product was cloned using the TA Cloning procedure (Invitrogen) and

sequenced on an ABI Prism 310 Genetic Analyzer (Applied Biosystems). We have also demonstrated that we can sequence not only normal monothio-ODN on the beads, **but also dithiophosphate-modified** bead-bound template sequences from a dithiophosphate combinatorial library. To our knowledge this is the first time that split synthesis has been used to create a "one-bead-one sequence" combinatorial library of oligonucleotides and that PCR was used to identify an ODN bound to a bead (**Yang et al., 2002, 2003; Gorenstein et al, US & Foreign Patents pending, 2002, 2003**).

Alhough the beads were screened against a target protein labeled with a fluorescent dye (Figure 11), the beads have also been screened directly against cell extracts. The binding of the NF-κB to a specific sequence can be detected using a primary anti-NF-κB Ab such as anti-P50 (Rabbit IgG Ab, Santa Cruz Biotechnology, Inc.) followed by a secondary Ab conjugated with Alexa Fluor 488 (goat anti-rabbit IgG from Molecular Probes). We have screened XBY-6 beads against WI-38 VA13, an SV40 virus-transformed human fibroblastic cell line extract, by similar fluorescence microscopy.

Ciphergen SELDI MS Detection of NF-κB bound to Thioaptamer Surfaces and Beads In partnership with Ciphergen and AptaMed (a UTMB spinoff of our DARPA technology) and funded in part through a U01 partnership grant to UTMB (D. Gorenstein, PI), we demonstrated the ability of our thioaptamers to bind both purified, recombinant NF-κB p50 and nuclear extracts on either beads (or ProteinChip surfaces). Duplex aptamers with a 5'-amino terminus linked to a 12 carbon chain was synthesized on our synthesizer. These were the dithioate 14-mers XBY-6 (C12-XBY-6), the normal phosphate backbone 22-mer NF-κB binding site with the C12 5'-amino linker (C12-IgκB) or a non-specific, non-covalently linked duplex (polydIdC) as a control. In Figure 12 we demonstrate that XBY-6 thioaptamer can capture recombinant p50 (MW ~ 46,200) on gel beads to which the 5'amino-C12 linked XBY6 is coupled to 20 um (1:1) AminoLink® Plus Coupling gel (Pierce). 3 μg of C12-XBY6 was coupled overnight at 4°C following the kit protocol. After quenching the gel, 6 μg of p50 in 1X EMSA buffer with polydIdC was added to the gel and incubated for 2 hrs with shaking at rt. The gel was washed to remove nonspecifically bound proteins, followed by one quick rinse with water. Protein bound to the gel was extracted with 5 μl of organic solvent (50% AcN and 0.01% TFA) with shaking for 20 min. All of the extracts were spotted onto NP20 ProteinChips, dried, followed by addition of saturated SPA and read on the Ciphergen PBSII MS (top two spectra). After extraction, 1 μl of the gel was loaded onto NP20 chip (bottom two spectra). Proteins still bound to the gel was analyzed using saturated SPA on the PBSII. Once again we show that p50 can be identified by SELDI, both in the extract and retained directly on the beads. In other spectra with more stringent washing, we can show that the XBY-6 spot but not the IgκB spot retains the bound p50 (spectra not shown), confirming the tighter binding of p50 to XBY6 (sub-nM) relative to IgκB (K_D 4 nM).



Figures 12 Detection of recombinant p50 on gel beads using XBY6. Top two SELDI MS extract from beads spotted onto NP20 ProteinChip. Bottom two SELDI spectra taken on beads themselves. Control no XBY6 covalently attached to beads with aminolinker .

Listing of all publications and technical reports supported under this grant or contract.

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(e) Technical reports submitted to ARO Monthly Progress Reports to DAPA

(7) List of all participating scientific personnel showing any advanced degrees earned by them while employed on the project

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