APTAMER SELECTION FOR JX-594

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Abstract

Aptamers are short oligonucleotide sequences that recognize and bind to particular targets. These targets can range from isolated proteins to targeted cell-surface receptors. Aptamers have similar properties to antibodies but can be selected for various affinities and are simpler and cheaper to produce, thus giving a greater versatility of targets and applications. Here, aptamers are being selected for Vaccinia Virus, an oncolytic virus currently under investigation for its use in targeting tumour cells. Selection of Vaccinia-binding aptamers will allow for better purification of the virus during manufacture and protection of the virus *in vivo* (Aptamer-Facilitated Virus Protection or AptaViP), allowing it to reach the tumour site without being eliminated by the body's immune system. To date, three potential pools of Vaccinia Virus binding aptamers have been identified through nine rounds of selection using a modified SELEX method adapted for use with small viral targets. Binding of the selected aptamers to the virus has been tested using flow cytometry and, in the best assays, a twofold increase in binding over three rounds of selection was observed. The highest binding pools of aptamers were used for three rounds of further selection. These increased the ability of the aptamer pools to bind the virus and they can now be used for cloning and investigation of functionality and future applications.

Acknowledgements

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Dr. Maxim V. Berezovski, PhD. (Supervisor)
Dr. John C. Bell, PhD. (Co-supervisor)

Dominique Vaillant, Research Technician
Chantal Lemay, PhD Candidate
Darija Muharemagic, MSc. Candidate

Berezovski and Bell lab groups

Jennerex Biotherapeutics

Table of Contents

Table of Figures

Contribution Statement

Conception:

My supervisor, Dr. Maxim Berezovski, along with my co-supervisor, Dr. John Bell, was responsible for the initial conception of the project. Experimental design for selection was based on previous work in the literature and modified by Dominique Vaillant (research technician in Dr. Bell's lab) and myself. Further experimental design was done by me in collaboration with Drs. Berezovski and Bell.

Writing:

This thesis was written in full by me with minor edits and revisions by Dr. Berezovski.

Experiments:

JX-594, the poxvirus used in this work, was produced by Dr. Bell's group. The N40 aptamer library has been used by our lab in previous work. Chantal Lemay (PhD candidate in Dr. Bell's lab) performed the flow cytometry (however, I prepared all the samples) and greatly assisted with data analysis. All other work was done by me.

Introduction

In a world that is rapidly breaking down boundaries and advancing to new frontiers, cancer remains an age-old enemy that has yet to be conquered. Despite tremendous advances in medical research, cancer remains one of the deadliest classes of diseases and is a leading cause of death in North America. Cancer comes in many types and as such, is referred to as a class of diseases rather than a single disease. In a nutshell, it is characterized by out of control cell growth whereby a cell loses control of its replicative mechanisms and proliferates without checkpoint control (1). Loss of control can occur for a wide spectrum of reasons. Genetic factors can play a role in up- or down-regulating key actors in the cell cycle. In addition, environmental effects, such as exposure to radiation or DNA damaging chemical reagents can also influence gene expression as well as cancer formation and progression. Current therapies include chemo and radiation therapy, surgery and treatment with immunological agents (interferon, antibodies, etc.). These have various degrees of effectiveness, depending on the cancer's stage, type, malignancy and other factors influencing the patient's health.

A novel treatment that is emerging for the treatment of cancers is the use of oncolytic viruses. These are viruses that have a natural ability to preferentially target and replicate in tumour cells, leaving healthy cells intact (2). Numerous viruses have been identified as having this ability, including strains of Newcastle disease virus, types of Parapoxvirus (particularly Orf virus), various adenoviruses and Rhabdoviridae, especially Vesicular Stomatitis Virus (VSV) (3). The latter has proven particularly efficient at reducing tumour size when used in either intratumoural or intravenous injections. Much of its specificity to tumour cells is owed to its high sensitivity to interferon signalling,

cytokines involved in the body's antiviral response, and this signalling pathway is down-regulated in many tumours (4; 5). Many of these viruses have also been genetically engineered in order to enhance their natural properties and reduce the risk of infection in normal, non-cancerous cells (5). Overall, oncolytic viruses are proving to be highly effective at treating solid tumours and some have entered clinical trials or have been approved for use as therapeutics (6).

Among the oncolytic viruses currently being developed, Vaccinia Virus is emerging as a leading candidate. This large, double-stranded DNA virus belongs to the pox family of viruses and has been used therapeutically in various ways. Most notably, injections of live Vaccinia Virus were used for smallpox inoculation throughout the 1960s and 1970s, leading to the worldwide eradication of the disease in 1977 (7). Since then, Vaccinia virus has been investigated for use in gene therapy, immunization against unrelated diseases (8), and, most recently, oncolysis. Wild-type Vaccinia virus has been used to treat cancers, in particular melanoma with 40% of injected patients showing no recurrence of the disease over two to three years in one study (9). However, specific mutations induced in Vaccinia virus have been used to render it more efficient. One such mutation is the deletion of Vaccinia Growth Factor (VGF) which mimics Epithelial Growth Factor (EGF) and induces cells surrounding the infection site to adopt a proliferative state, making them more susceptible to infection (10). Without this growth factor, the Vaccinia's ability to rapidly infect host cells is reduced. A second mutation involves the deletion of thymidine kinase, a stimulant of cellular growth involved nucleotide synthesis (10). Deletion of this kinase significantly reduces the virus' virulence towards normal, untransformed cells (8). However, in cancer cells, the EGFR pathway is upregulated (11) and the cells are already in

a highly proliferative state, increasing their susceptibility to Vaccinia virus. In addition, the pathways stimulating cellular production of thymidine kinase are upregulated in 90% of cancers, meaning that in these cells, the virus can rely on the host's enzyme rather than its own (10).

Various feats of genetic engineering have led to the development of other variations of Vaccinia virus. One such is JX-594 which contains the thymidine kinase deletion outlined above. However, in this case, the gene has been replaced by another gene which expresses human granulocyte macrophage colony stimulating factor (hGM-CSF). This cytokine has already been used in injected form to stimulate the body's immune system to target tumours enhancing the body's ability to suppress tumour growth (12). JX-594 has been shown to successfully express GM-CSF and is associated with the induction of tumour-specific cytotoxic T lymphocytes (13). Clinical trials for therapeutic use of JX-594 against a number of cancers, particularly melanoma and hepatocellular carcinoma, are underway and results thus far are positive, though conclusive trial results are still pending (14).

Promising though the use of JX-594 has so far proven, a number of challenges associated with its production and use have been identified. One such challenge is the potential for the development over time of an immune response against the virus leading to decreased oncolytic activity as the virus gets eliminated from the blood stream. This is particularly concerning owing to the ability of the immune system to generate antibodies against a given challenge even decades after originally encountering it, as is the case with many individuals vaccinated against smallpox (7). Though this problem has so far been absent during treatments of short to medium duration (15), long term effects are not yet

known. Particularly when one considers that a significant portion of the population has been inoculated against smallpox using Vaccinia, the problems arising from long-term acquired immunity could be significant. Problems could also arise in cases of tumour regression. A second challenge lies in the efficient purification of the virus during manufacture. As JX-594 expands in its use as a therapeutic agent, its mass production as a highly purified and clean product will become a necessity. The generation of an easy technique for viral purification will also increase the efficiency and effectiveness of the research still being conducted both on JX-594 and other variations of Vaccinia virus. At present, many of the techniques that allow for rapid purification of the virus tend to leave behind a significant amount of cellular debris from the production process, generating a high titer of virus but a low grade of purity. Obtaining highly purified products currently requires a significant amount of time and effort. In this project, the use of aptamers as a potential remedy to these challenges is being proposed.

Aptamers are very short oligonucleotides, generally 80-100nt in length, composed of RNA or ssDNA. Their most prominent characteristic is their ability to adopt a particular three-dimensional structure, determined by their primary sequence, which will recognize and bind to well defined targets (16). A wide variety of targets are possible, with some aptamers recognizing cell surface receptors, while others recognize small molecules such as theophylline (17) . They are generally selected from a large library through a process known as SELEX – Systematic Evolution of Ligands through Exponential Evolution. A variety of protocols have been developed, including cell-SELEX, in which a pool of aptamers is exposed first to a cell line lacking the desired target to remove unspecific aptamers, then to a cell line containing the target to identify an aptamer that will

bind. These aptamer pools can be amplified by PCR and the resultant pool be used for another identical round of selection which will reduce the number of different aptamers present in the population, but increase the number of those that bind to the target. In this way, a pool of highly specific aptamers is gradually evolved from an initial randomized library (18). These aptamers can then be used for a host of downstream application. In some cases, they have been used as riboswitches that, when bound to their ligand, adopt a DNA-binding conformation that inhibits gene expression (19). They have also been used to facilitate biomarker discovery and recognition (20), as probes for intracellular protein localization (21) and hold promise for their use in the field of personalized medicine (22).

A particular challenge when working with aptamers is the tendency of certain sequences to be "sticky" and bind non-specifically to a wide variety of targets (23). This ability means that within any given pool, it is difficult to determine with certainty that aptamers are binding the desired target and not the materials being used, leftover cell debris or any other surface with which they may come into contact. This increases the importance of a strong negative selection step capable of eliminating these aptamers from the candidate pools. However, one advantage to working with aptamers is the potential for selecting multiple aptamers to the same target, but with various affinities and specificities to different surface proteins on the virus. This provides aptamers a decided advantage over antibodies in their use. In addition, being nucleotides, aptamers can be repeatedly denatured and renatured with relative ease (24).

Here aptamers are being selected against JX-594 using an N40 library. Numerous types of aptamers exist, both with fully randomized and with partially structured sequences (25). This particular library is 80 nucleotides in length and contains two flanking primer

regions of 20nt each and a central region of 40 randomized nucleotides, allowing for a large number of possible aptamers. The primers used for the amplification of these aptamers are labeled with Alexa Fluor 488, a fluorescent dye with an emission maximum at 514nm, which allows for their detection either alone or when bound to their target using common fluorescence imaging techniques. Owing to the small size of the desired target, the normal selection procedure had to be modified in order to work with viral particles, as will be discussed hereafter.

The aim of this work is to select aptamers that bind JX-594. The objective in this is twofold. The first is to develop a protocol for effectively and simply selecting aptamers against a small viral target. The second is to begin addressing some of the challenges associated with the use of JX-594 as an oncolytic virus. An aptamer binding JX-594 with a high affinity could assist with the purification of the virus following *in vitro* manufacture. Currently, heparin columns and centrifugation through a sucrose cushion are used for purification. As stated above, these have not proven effective at eliminating remnants of cell debris from the preparation. A column coated with an aptamer, highly specific to the virus could prove capable of separating JX-594 from contaminants and a simple change of pH – thus denaturing the aptamer – would then release complex. The other challenge that has been outlined involves the *in vivo* survival of the virus once injected into a patient. An aptamer-based shield used to coat the virus would protect it from the effects of neutralizing antibodies and allow it to reach the tumour site intact. This Aptamer-facilitated Virus Protection (or AptaViP as it has been termed) could greatly enhance the effectiveness of JX-594 as a treatment for various forms of cancer. Because aptamers can be selectively modified, they can be protected from the effects of nucleases and other degradation

enzymes in bodily fluids. Thus, the addition of methyl groups, RNA caps and other chemically induced modifications allow the aptamers themselves to survive *in vivo* (26). It can also be possible to select aptamers against various epitopes on the virus, thus blocking a variety of antibodies. Though it is feasible to select aptamers against those antibodies and to use them to neutralize the immune response, it is far more efficient to shield the injected virus (nonetheless, this latter approach is being investigated by our group). In both cases it is feasible that different aptamers would be necessary for each patient since a variety of epitopes may be recognized by antibodies. A structure for JX-594 binding aptamers could be developed with variations being inserted as necessary.

Though the work being undertaken is still at the first stage – that of selecting JX-594 specific aptamers – results so far are proving promising. This line of investigation, at the threshold between cancer therapeutics, personalized medicine and drug based delivery systems, is contributing to the development of cutting edge medical technologies and to the fight against age old disease.

Materials and Methods

Choice of DNA aptamer library:

DNA N40 library was used in all experiments. This consists of single-stranded DNA oligonucleotides, 80 nucleotides in length. Two flanking primer regions, 20 nucleotides long each, surround the central aptamer region. This is formed by 40 randomized nucleotides, given a large diversity of potential aptamers. Thus, library sequence is GAG GAG ACT GAC ATT GGT GC - N (40) - GC ATA GGT AGT CCA GAA GCC. Aptamer libraries as well as primers (see PCR, below) were prepared by Integrated DNA Technologies Inc.

Selection of aptamers using centrifugal filter unit:

10^6 pfu of JX-594 were incubated with 100nM of N40 DNA library in 100µL of PBS + 5mM Mg^{2+} for 30 minutes at 4 and 37°C. Incubation mixture was then diluted to 500µL with PBS + 5mM Mg^{2+} loaded onto a 100kDa centrifugal unity (Millipore). Mixture was centrifuged at 10 000 rpm for 10 minutes, washed with 500µL PBS + 5mM Mg^{2+} and centrifuged again at 10 000 rpm. Complex was collected by inversing the filter and spinning at 6 000rpm for 2 minutes. Mixture was heated at 95°C for 5 minutes to inactivate virus and dissociate complex. DNA was then amplified by PCR. Further cycles were repeated to select aptamer clones.

Selection of aptamers using centrifugation:

10^6 pfu of JX-594 were incubated with 100nM of N40 DNA library in 100µL of PBS + 5mM Mg^{2+} for 30 minutes at 37°C. Mixture was centrifuged at 17 200rcf for 20 minutes. Supernatant was removed and pellet resuspended in 100µL of PBS + 5mM Mg^{2+} then

transferred to a new tube and centrifuged again for 10 minutes. Wash step was repeated a second time. Virus-aptamer complex was heated at 95°C for 5 minutes to inactivate the virus and dissociate the complex. DNA was amplified by PCR for use in further cycles.

PCR:

Polymerase Chain Reaction was performed using GoTaq® Hot Start Polymerase kit (Promega) according to manufacturer's specifications. Reaction mixture contained 2.5mM $MgCl_2$, 200µM dNTP mix (Promega), 0.025u/µL polymerase and primer mixes as indicated below. PCR was performed using 15 cycles symmetric PCR followed by 20 cycles of asymmetric PCR. Symmetric PCR was performed using F4 forward primer (5'-CTC CTC TGA CTG TAA CCA CG-3') and R4 reverse primer (5'-GGC TTC TGG ACT ACC TAT GC-3') at a concentration of 300nM each. For asymmetric PCR, Alexa-488-labelled F4 primer was used at a concentration of 1µM along with 50nM R4 primer. Annealing temperature was 56°C.

Aptamer purification:

Following amplification, aptamers were purified by incubation in a buffer composed of 50mM Tris-Acetate (pH 8.4), 50mM NaCl and 5mM $MgCl_2$. Mixture was loaded on a 30kDa cut-off filter (NanoSep) and centrifuged at 5 000rpm for 20 minutes, then washed twice in 200µL incubation and centrifuged for 10 minutes each time. Purification test was performed by running raw and purified samples on a 3% agar gel at 160V to check for the presence of primer bands.

Flow cytometry:

10^7pfu of JX-594 were incubated in 100nM of each pool to be tested for 30 minutes at the same temperature used for selection. Buffer used was PBS + 5mM $MgCl_2$. 0.1g/mL of Hoeschst 33342 dye was used as a positive control to stain intra-viral DNA and mark the presence of virus particles. Samples were analyzed using a CyAn Flow Cytometer (Beckman Coulter) using a FITC laser (FL1 channel) to detect Alexa 488. Hoeschst was detected in the FL6 channel and forward scatter used to determine particle number. Data was analyzed using Kaluza or FlowJo software.

Results

Development of selection protocol:

The first task in the selection process was to identify and design a functional protocol for the selection of aptamers against small viral targets. As outline above, the SELEX procedure developed for cellular targets needed to be modified in order to suit our requirements. When the target, whether viral or cellular, is incubated in a pool of potential aptamers, those with the highest affinity to the target will bind while the remainder remains free in solution. The challenge lies in the efficient separation of the virus-aptamer complex from the unbound aptamers. A procedure was developed in which the complex was passed through a syringe filter, but it was found that this generated significant product loss (previous work from summer; results not shown). Eventually, it was determined that the use of a centrifugal filter unit, large enough to allow free aptamers to flow through, but small enough to retain the virus, would be the most efficient option. Figure 1 outlines the general procedure that was adopted for rounds 1-9 of selection. Starting with an initial pool of the N40 DNA library, a negative selection was performed against the filter, a necessary step owing to the propensity of aptamers to bind to such a wide variety of targets as cellulose and polypropylene as well as the intended target. Next, the remaining aptamers were incubated with JX-594 for 30 minutes at either 4°C (since the virus is more stable at that temperature) or 37°C (a far more useful temperature for *in vivo* use). Aptamers expected from each condition would be different since the same aptamers can feasibly adopt a different conformation at each temperature. Separation using a centrifugal filter yielded aptamer-virus complex caught on the filter which could be recuperated. The complex was easily dissociated by heating to 95°C in order to inactivate JX-594 and

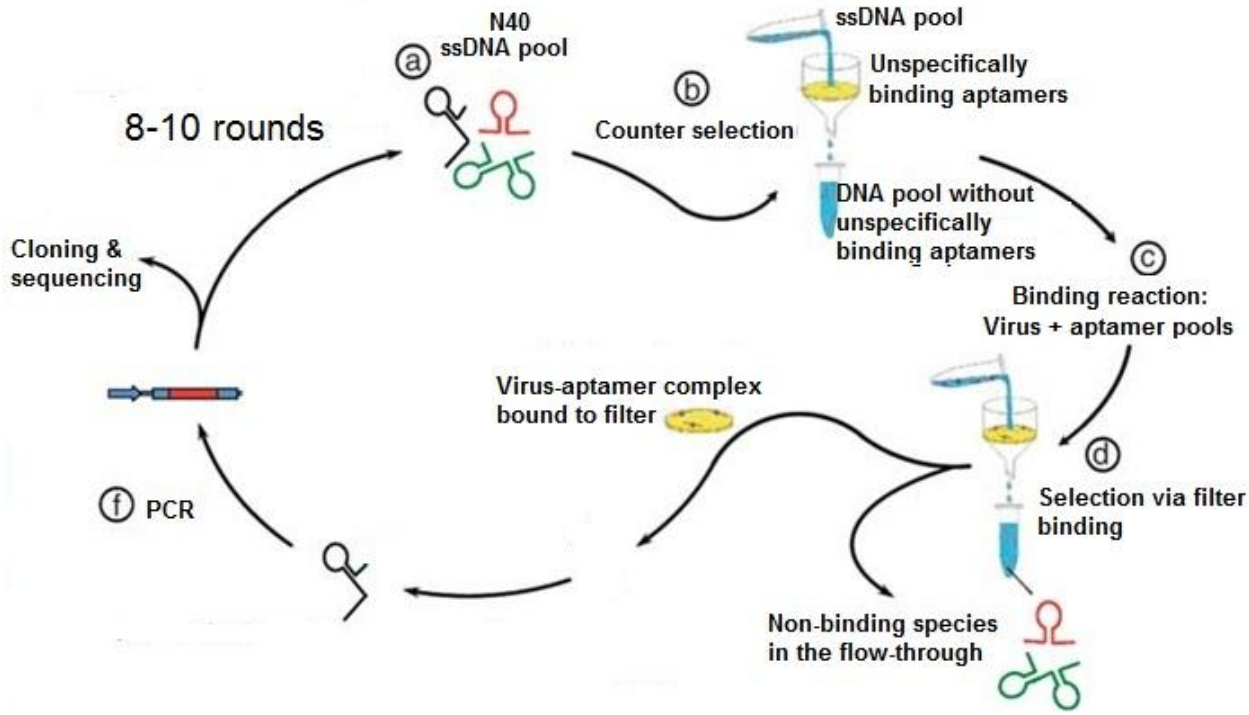


Figure 1. Strategy for selection of aptamers against viral targets. N40 aptamer pool was used in counter selection against centrifugal filter units (Amicon Ultra-0.5) and then incubated with JX-594 at 37°C or 4°C for 30 minutes. Virus-aptamer complex was isolated using the centrifugal filter units and dissociated by heating at 95°C. Aptamers were then amplified by PCR and, following purification, used for further rounds of selection. Once a sufficient number of rounds have been performed (generally 8-10) aptamers can be cloned and sequenced for use in numerous downstream applications.

denature the DNA. Aptamers could then be amplified by PCR using a tandem symmetric/asymmetric approach in order to regenerate the single-stranded DNA pool. The enriched pool could then be used for successive rounds of selection before proceeding to cloning and sequencing of the highest binding aptamers.

Completion of initial rounds of aptamers selection:

Selection started using the method outlined above. Aptamers were incubated either with virus at 4 or 37°C or without virus at 4°C before being centrifuged using the filter of choice. After each round, the presence of aptamers was verified by gel electrophoresis (see Figure 2) in order to guard against product loss. In order to verify the efficacy of the negative selection, aptamers were incubated against the filter and in the absence of virus during each round of selection. As Figure 2 shows, aptamers selected in the absence of virus were always present suggesting the inclusion of non-specific aptamers in the pools being evolved. It was also clear that aptamers remained in the pools being selected against JX-594, though a comprehensive binding assay would be required in order to verify their specificity to the virus. Purification of the aptamers following amplification was efficient, yielding significant diminution of primers in the pools without corresponding to a large loss of the desired product. Nine rounds of selection were completed in this manner, ideally leading to the identification of a highly specific pool of aptamers that bind JX-594, though each of the aptamers in that pool may have a variety of affinities.

Verification of aptamer binding:

A number of methods were assayed to check that the selected pools of aptamers were indeed binding the virus (previous work from summer; results not shown). Because

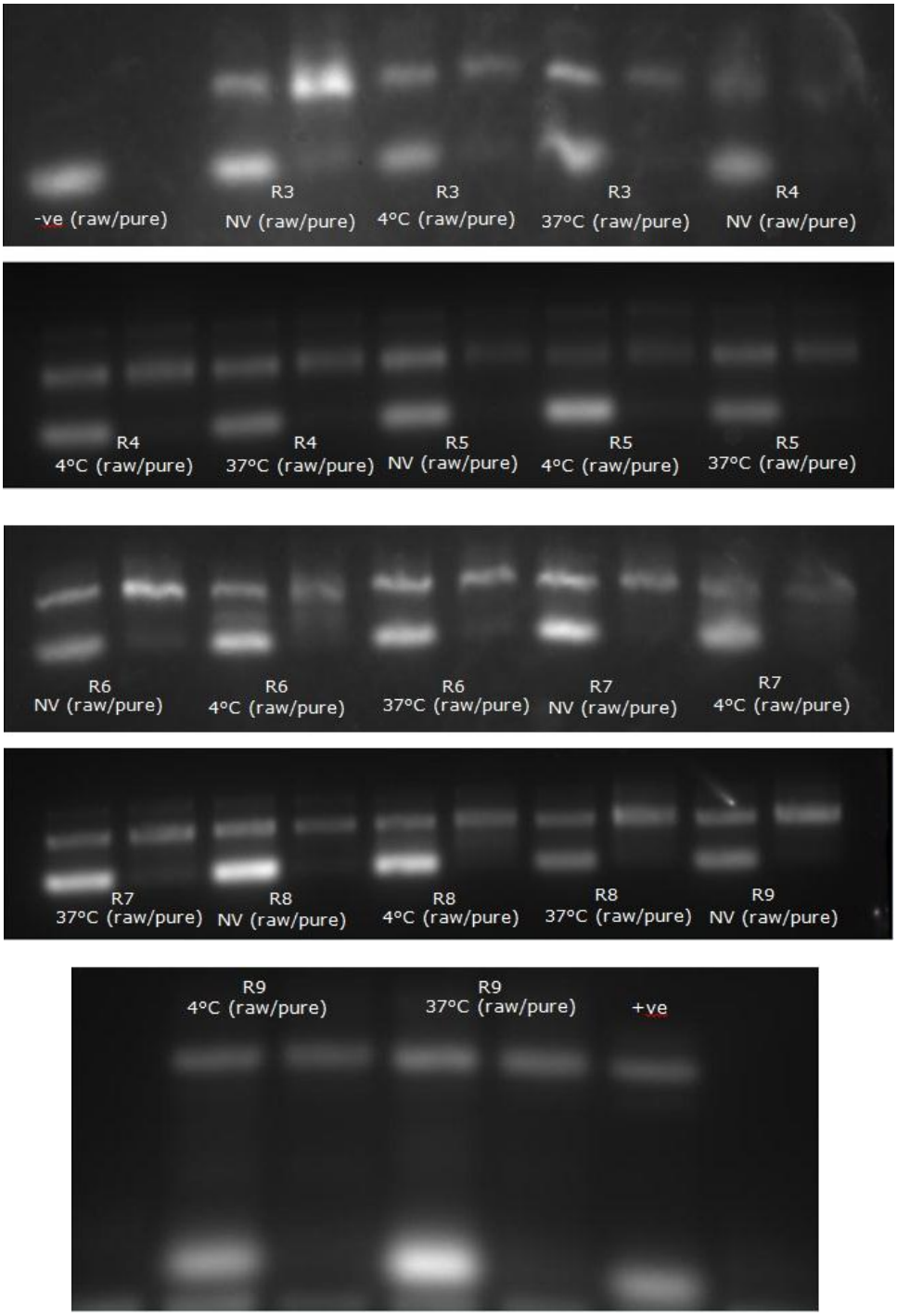


Figure 2. Aptamers selected against JX-594. PCR amplification of N40 DNA aptamer pools collected after incubation with (at 4°C or 37°C) or without (indicated by NV for no virus) 10^6pfu of JX-594. Individual rounds of selection are indicated by R*x*. Amplicons were run either directly from PCR mixture (raw, at left of each set) or after purification (pure, at right of each set). 15 cycles of symmetric PCR followed by 20 cycles of asymmetric PCR were performed and samples loaded onto a 3% agar gel and run at 160V for 20 minutes for electrophoresis separation. A water sample was used for negative control (-ve) while 10^6 molecules of N40 library were used as a positive control (+ve).

the forward primer used in amplification is labelled with Alexa Fluor-488 (AF-488), the most evident methods would involve the measurement of a fluorescent signal. The challenge lay in determining a method sensitive enough to track the fluorescence of a very small oligonucleotide. It was eventually determined that flow cytometry provided the best option. An initial run was performed to check whether the virus would appear in flow cytometry, to verify if a shift in AF-488 signal was evident when aptamers were included in the mixture and to begin ascertaining that binding was increasing in each round of selection (see Figure 3). It was evident that aptamers alone were too small to show significant signal in FL1 fluorescence. Anything that did appear in this assay could be attributed to background noise from the solution (Fig.3a). Next, it was determined that JX-594, when run on its own, was detectable in flow cytometry, mostly owing to the tendency of the virus to form aggregates (see discussion) (Fig.3b). This allowed a threshold to be set, outlining where the auto-fluorescence of the virus fell, above which a particle could be considered to be fluorescing and, thus, bound to an aptamers. A preliminary test using JX-594 incubated with the initial N40 library was run to determine the start point for aptamer selection. A small but clear shift in fluorescence was observed (Fig.3c).

With this in mind, a first test was performed to determine whether or not significant shifts in fluorescence could be detected following selection of aptamer pools (Figure 4). Aptamer pools from rounds 3, 6 and 8 were analyzed and compared to the appropriate controls. As seen in Figure 4a, the selected pools do in fact show a shift in fluorescence when compared to both the virus on its own and the virus incubated with the unselected library. However, a disturbing tendency was observed when the corresponding aptamer

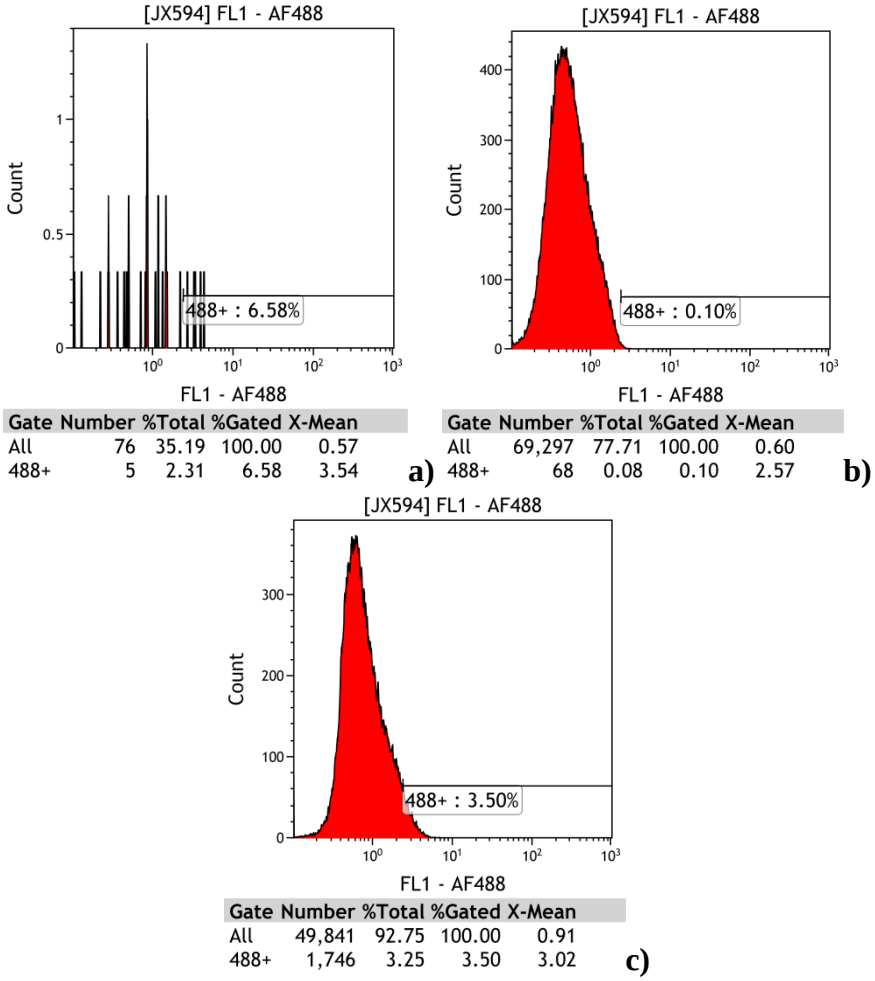
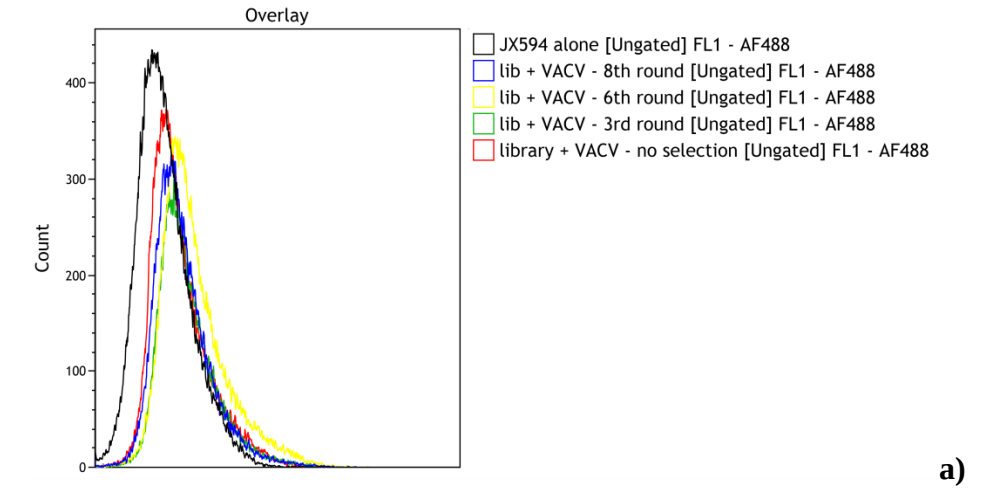
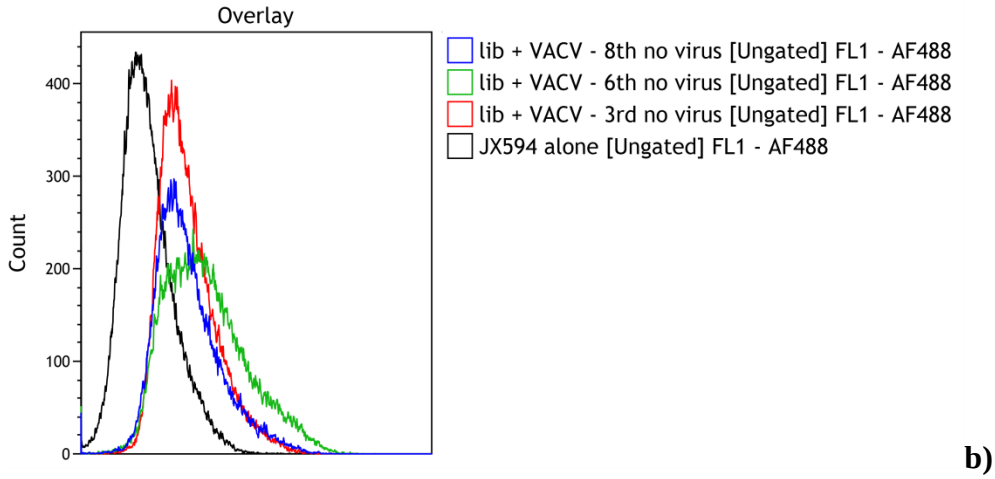


Figure 3. Definition of binding assay efficiency. CyAn (Beckman Coulter) flow cytometry analysis of N40 aptamers binding to JX-594. Events were detected on FL1 and histograms generated by plotting event count (linear) in function of relative fluorescence (on a log scale). A) Native N40 library run alone showing that unbound library does not appear in the resulting plot. B) Virus run alone as negative control allowing for the setting of a gate. C) JX-594 incubated with native N40 library to set baseline fluorescence without any aptamer selection.



a)



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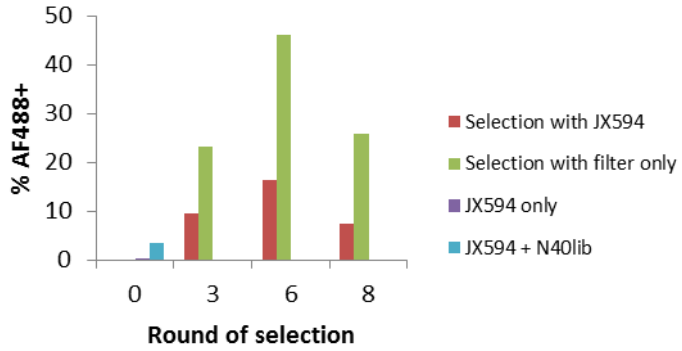
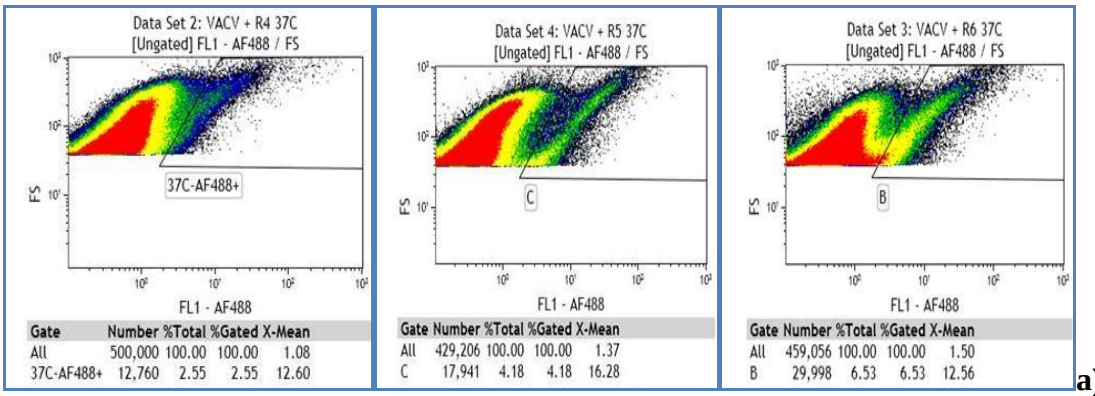


c)

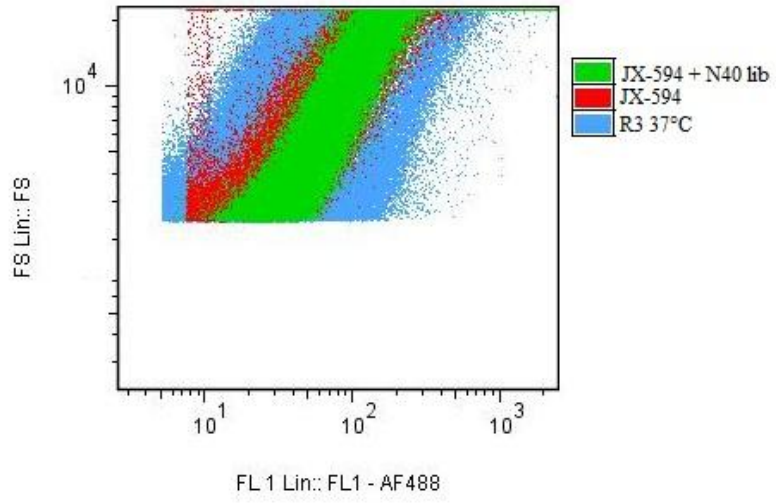
Figure 4. Initial test of aptamer binding to JX-594. (A and B) Composite overlay of Beckman Coulter CyAn analysis of N40 aptamers binding to JX-594 following each of the indicated rounds of selection. Events were detected on FL1 and histograms generated by plotting event count (linear) in function of relative fluorescence (on a log scale). A) Aptamers selected in the presence of virus showing a small increase in fluorescence in rounds 3 and 6 followed by a drop in round 8. B) Aptamers selected in the absence of virus (just against the filter) showing an even greater increase in fluorescence after 3 and 6 rounds of selection. A similar drop was observed after 8 rounds. C) Analysis of N40 aptamers binding to JX-594 following indicated rounds of selection. Percentage of Alexa-488 positive virus particles is indicated for virus alone, virus with native library and aptamers selected in the presence and absence of virus.

pools that were selected solely against the filter were incubated with the virus and analyzed (Fig. 4b). These showed an even greater shift in fluorescence when compared to the negative control. Figure 4c allows us to see that round 6 expressed the greatest level of fluorescence but that, in all rounds tested, aptamers selected in the absence of virus showed more binding than their counterparts selected directly against the virus. Though this was a cause for some concern, the experiment did allow us to conclude that flow cytometry analysis would enable the detection of aptamer binding to JX-594.

Further testing was then performed on all the selected pools in order to check their affinity with the virus. For each round, three conditions were analysed. In the first, aptamers incubated with the virus at 37°C were verified. Next, we used aptamers incubated with JX-594 at 4°C, an easier condition to maintain. It is noteworthy that controls (virus on its own and incubated with the intial unselected library) were also kept at this temperature. Lastly, aptamers incubated solely against the filter and in the absence of virus were used. The most promising data was obtained from the pools at 37°C. An increase in the population expresseing Alexa Fluor-488 fluorescence was observed over rounds 4 (2.55% of the population), 5 (4.18%) and 6 (6.53%) of selection (Fig. 5a), leading to the speculation that the aptamers were in fact becoming more and more specific to JX-594. The emergence of a side population, visible in flow cytometry analysis, made it clear that a change was occuring in the overall population being analyzed. This side population was not visible when the virus was analyzed alone nor when it was incubated with unselected library. These two populations showed a lower shift in fluorescence than the virus incubated with selected pools, though it covered a narower range (Fig. 5b). Figure 5c shows the combined data from all samples analyzed. Though aptamers selected at 4°C



a)



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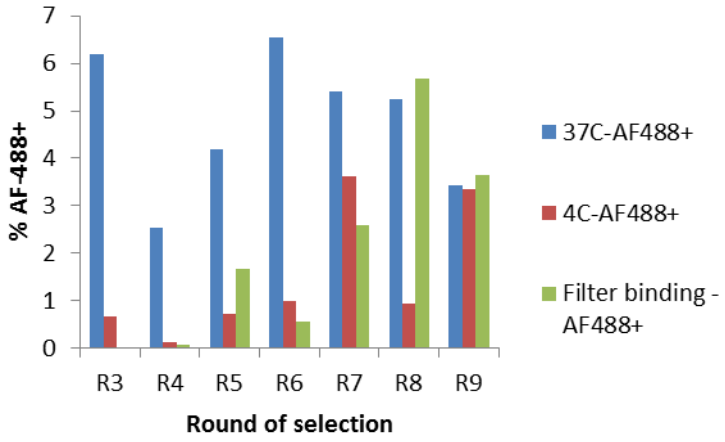

c)

Figure 5. Selected aptamers bind to JX-594. CyAn (Beckman Coulter) flow cytometry analysis of N40 aptamers binding to JX-594. Events were detected on FL1and graphs generated by plotting forward scatter in function of FL1 fluorescence on a log scale. A) Aptamers from rounds 4 through 6 of selection at 37°C showing the appearance of a higher fluorescing side population in round 4 (left), appearing larger in round 5 (centre) and even larger in round 6 (right). B) Overlay of control populations (JX-594 alone and with initial N40 library) with aptamer pool from round 3 at 37°C showing lower fluorescence in control pools. C) Analysis of N40 aptamers binding to JX-594 following indicated rounds of selection. Percentage of Alexa-488 positive virus particles is indicated for each round under each of the specified conditions.

showed some binding to the virus, it was clear that this was not at particularly high levels. For this reason, further work with these pools was discontinued. The aptamers selected against the filter originally showed no specificity for the virus, but by round 5 of selection it is clear that some portion of that population binds to JX-594 as well as to the filter, suggesting the presence of particularly "sticky" aptamers. From among the filter binding pools analyzed, the one with the highest affinity to the virus was from round 8. This pool of aptamers was therefore identified as meriting further investigation. Analysis of the pools selected at 37°C revealed that the pool from round 3 – the first analyzed by flow cytometry – showed higher binding than subsequent pools for reasons that remained unclear. This pool was also identified for further work. From there, specificity of the aptamer for JX-594 increased round after round until round 7 where it began to decrease. This trend is consitent with results observed by others (see discussion) and allowed us to identify the pool from round 6 as the best candidate for containing aptamers binding JX-594. This pool became the third candidate chosen for further investigation.

Subsequent rounds of selection:

Having identified three potential pools of aptamers that bind to JX-594, it was decided that further selection should be done on each of these pools in order to increase their specificity for the virus. In addition, in an effort to reduce the number of aptamers with non-specific binding, the selection method was changed for the following three rounds of selection. Rather than separate the virus-aptamer complex using a centrifugal filter, the complex was pelleted using centrifugation while the unbound aptamers remained free in solution. The hope was that aptamers which may have bound to the filter would not also bind to the centrifugal tube used or could be eliminated by extensive washing. As can be

seen in Figure 6 however, eliminating all non-specific aptamers still proved to be an elusive goal. Nonetheless, three rounds of selection were performed using this method. Figure 6 also makes it clear that the selection and amplification of aptamers was still possible using this method. Reducing the non-specific aptamers in any given pool will no doubt require rigorous binding assays as well as analysis of individual aptamers following cloning.

Once the three further rounds of selection had been performed, a final binding assay was performed to check for increased binding to the virus. Though the shift in fluorescence in each of the three rounds was slight, it was clear that the final pools still represented a shift in fluorescence when compared to the virus on its own (Fig. 7a). In fact, 8.59% of the R3-3 (initial pool: round 3; continued selection: round 3) pool was aptamer positive, 14.8% of the R6-3 pool and 21.8% of the R8-3 pool. However, owing to the fact that the R8-3 pool was, in the initial selection, a filter-binding pool, use of this pool would first be dependent on analysis of individual clones to verify their specificity to the virus. In these continued rounds of selection, aptamers were again incubated in the absence of virus. These pools also showed expression in FL1. However, when overlaid with controls (JX-594 alone and with initial N40 library), it was clear that the shifts from R3-3 and R6-3 were not significant when compared to the virus on its own, suggesting that very little binding occurred. As for the pool taken from round 8 of the initial selection, it fluoresced similarly to the initial library on its own, suggesting that both contained highly unspecific aptamers that may bind to the virus, but do not do so exclusively (Fig. 7b). This allowed us to conclude that we were dealing with three pools of JX-594 aptamers which can now be used for cloning and sequencing before embarking on downstream applications.

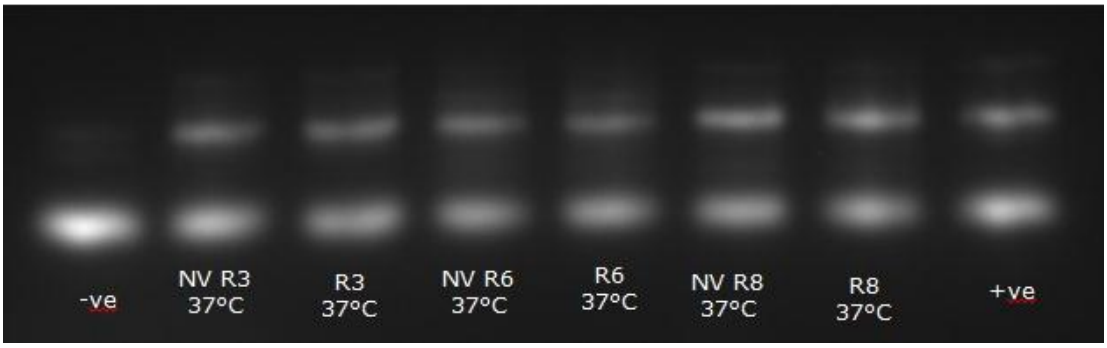

Figure 6. Subsequent selection of aptamers binding JX-594. PCR amplification of N40 DNA aptamer pools collected after incubation with or without (indicated by NV for no virus) 10^6pfu of JX-594 at 37°C. Original pool (from initial rounds of selection) is indicated by R*x*. 3 rounds of selection were performed using the centrifugation strategy. After each round, 15 cycles of symmetric PCR followed by 20 cycles of asymmetric PCR were performed and samples loaded onto a 3% agar gel and run at 160V for 20 minutes for electrophoresis separation. A water sample was used for negative control (-ve) while 10^6 molecules of N40 library were used as a positive control (+ve). Representative samples are shown here.

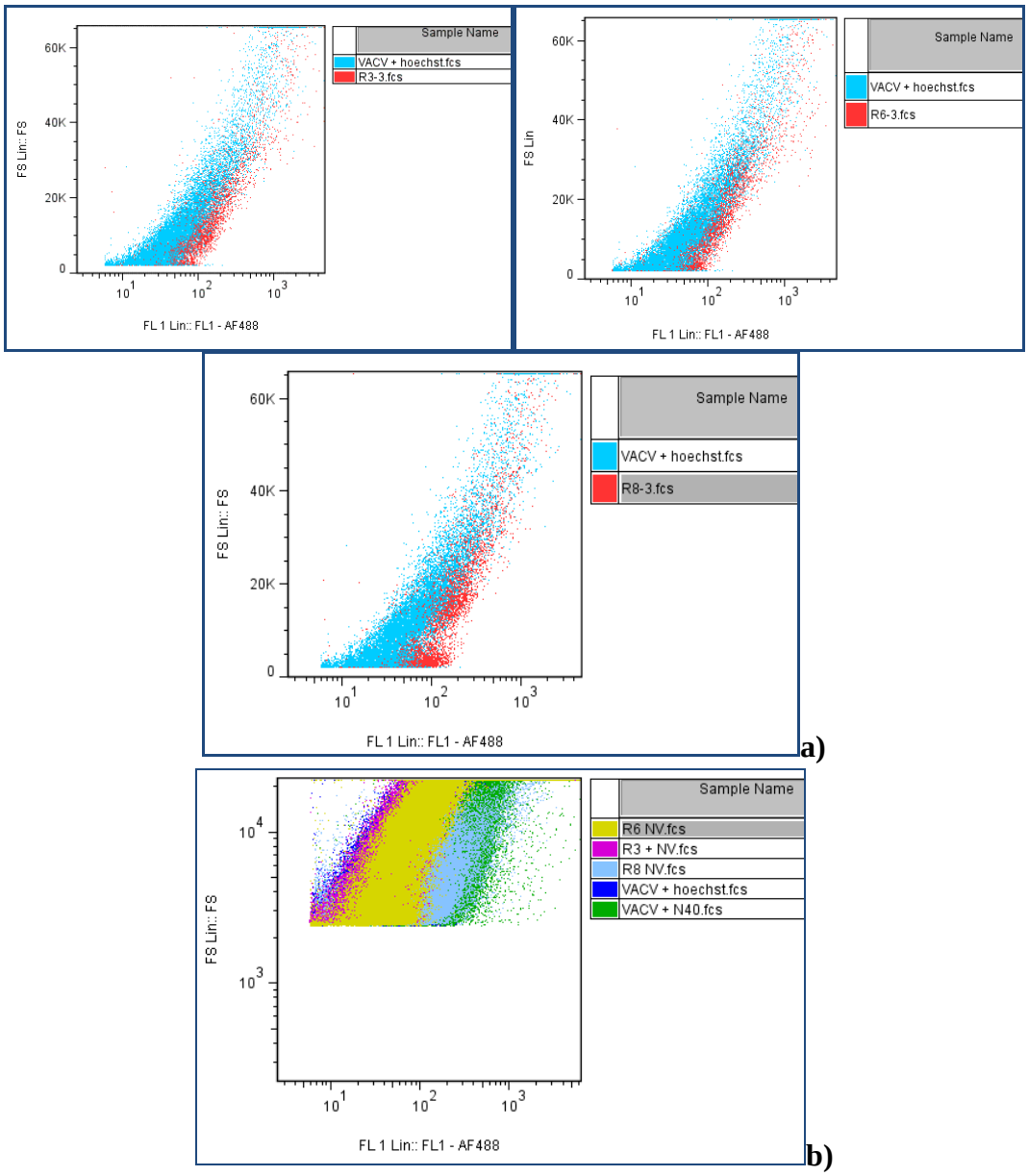

Figure 7. Selection of JX-594 aptamer binding pools. CyAn (Beckman Coulter) flow cytometry analysis of N40 aptamers binding to JX-594. Events were detected on FL1and graphs generated by plotting forward scatter in function of FL1 fluorescence. A) Aptamers taken from the initial selection in round 3 (left), round 6 (right) and round 8 (bottom) were evolved over three more rounds of selection with JX-594. Overlay is shown with JX-594 alone (in blue) and the identified pool (in red) showing a shift in fluorescence in FL1. B) Overlay of controls showing virus alone (dark blue), aptamers from round 3 of initial selection with continued selection in the absence of virus (pink) and aptamers from round 6 selected in absence of virus (yellow) fluorescing in the same area. Meanwhile, unselected N40 library (green) and aptamers from round 8 selected in absence of virus (light blue) fluoresce in the same range.

Discussion

A number of questions naturally emerged from this work, many concerning the methods employed. For example, though aptamers can be selected at any temperature desired, it is usual practise when working with cellular target to do it at a low temperature (generally 4°C) in order to reduce the risk of DNA internalization by the cell (18). However, so far as literature reviews could determine, this does not appear to be a concern when working with a virus (which is normally internalized, rather than doing any internalizing). On the other hand, Vaccinia virus itself is less stable at higher temperatures with a significant decrease in titer apparent when it is kept for too long at 37°C (27; 28; 29). This is worth taking in note as it could have a role in skewing results obtained from these experiments. However, because we are merely selecting aptamers that bind to the outer envelope of the virus, it is likely that binding can still occur even if the particle is dead and that this does not dramatically affect the experiments.

Another challenge associated with working with this virus is the tendency for Vaccinia to form aggregates, particularly following repeated cycles of freeze/thaw, common when performing multiple experiments (30). This not only makes it challenging to determine the titer with any certainty, it also renders it difficult to use easily for infection. However, it does have the advantage of rendering it more visible in flow cytometry. Generally, viruses are too small to be visible to a flow cytometer, though recent work using highly fluorescent dyes (such as SYBR Green) has begun to show otherwise (31). However, the aggregates that form are in fact large enough to be detected, thus allowing binding assays to proceed with some degree of accuracy.

One essential step in this work is being able to efficiently eliminate non-specific aptamers using a negative selection step. In the first method of separation, this was done by passing the aptamer pools through the filter and only retaining those that did not bind to its surface. However, when selection was done by centrifugation, a negative selection step was no longer possible and only extensive washing could diminish the possibility of aptamers binding to the tube walls. As results showed however, neither method proved completely effective at eliminating non-binding aptamers. Flow cytometry analysis, particularly that shown in Figure 7, does highlight that many of the unspecific pools fluoresce with a similar pattern as the control samples. This would suggest then that cloning individual aptamers is necessary in order to identify which aptamers in the pool bind exclusively the virus and which also bind other targets, but that using both selection methods in combination may reduce the number of unspecific binders that are being analysed. In this way, aptamers with 'sticky' sequences can be identified (23) and eliminated from the pool.

Determining the ability of a particular pool of aptamers to bind to JX-594 required a strong and reliable binding assay. In this case flow cytometry proved capable of visualizing the virus and the shift in fluorescence when aptamers bound it. However, certain challenges remain. The first is the lack of a strong positive control. One method was tried using Hoeschst 33342 dye which can penetrate the viral envelope and stain the internal DNA of the virus particles (data not shown) (32). This would allow us to distinguish between JX-594 and other cell debris, assuring that we were indeed working with virus particles. However, it was eventually recognized that Hoeschst can dye particles remaining in the extracellular matrix as well as internal DNA (33) and that this would

therefore not be an effective way to ascertain the presence of JX-594 virus. The ideal control would be a Vaccinia binding antibody. Though antibodies recognizing Vaccinia envelope proteins exist and have been used before (34), during this project, none were available that were conjugated to a fluorophore. This would be necessary for its use in flow cytometry and would have provided an ideal positive control, allowing us to see what a strongly fluorescing JX-594 population resembles.

Another problem emerged from the high auto-fluorescence of the virus when analysed alone. This creates challenges when trying to compare potential binding aptamers to the virus alone since the shift in fluorescence is less distinguishable. A couple different strategies could be used in future to eliminate this problem. One suggestion has been to analyze data both in the FL1 and the Fl2 channels. Compensation with these two channels can eliminate crosstalk between them (35; 36). Compensating should then allow us to distinguish between fluorescence that is only present in FL1 (real fluorescence of AF488) and fluorescence that is equally present in FL2 (auto-fluorescence of the sample). This will require re-analysis of all samples since the FL2 channel was not used in these experiments.

In addition, there are suggestions that baseline fluorescence of biological samples increase when heated (37-38). If it is possible that this is occurring with JX-594, then the importance of ensuring that controls are the same temperature as the samples increases so that baseline fluorescence is comparable and false shifts are not identified. Due to the fact that this was not the case in the experiments shown in Figure 5, fluorescence shift has been compared to other samples, rather than exclusively to the virus alone. For this reason, trends of increasing fluorescence have been identified along with simple measures of fluorescence.

One final suggestion related to reducing autofluorescence of the virus was made. It has been determined that biological samples have the highest autofluorescence at wavelengths under 500nm and weakest fluorescence at those above 600nm (39). Unfortunately, the high autofluorescence range corresponds to the emission peak of Alexa-488, the dye being used for visualization. One option then is to change the fluorophore label of the primers, perhaps using Alexa Fluor 647 instead which emits well into the 600nm range.

A final problem we observed in the first analysis of aptamers was the decrease in binding efficiency following round 6 of selection at 37°C (see Figure 5). Although worrisome, others have also reported a decrease in the capacity of aptamers pools to bind their targets following numerous rounds of selection (40; 41). A number of observations and possible mechanisms have been identified relating to this phenomenon, including randomized amplification of unspecific aptamers from a given pool, but the phenomenon has not been adequately explained. We have compensated for it by selecting the highest binding pool and continuing selection based on this. Effectively, later results showed that the decrease in selection was eliminated by taking this approach.

As such, the aptamers obtained in the final pools analysed, particularly those from the R6-3 round, have a high probability of containing a number of aptamers that will bind to JX-594. Cloning and sequencing of these aptamers will allow for a more thorough analysis of these aptamer sequences. Inclusion of some of the specific suggestions outlined above will help to refine the results. Following this, kinetics of the aptamer-virus complex can be determined. The method commonly used in our lab for accomplishing this is capillary electrophoresis. Analysis of resulting electropherograms allows the resolution of

various affinity constants and determination of binding parameters. Mass spectrometry analysis can be used to determine which surface proteins the selected aptamers bind. An aptamer binding with a high affinity would make a strong candidate for use in a purification column. Cell culture studies can be performed to determine the effect of each aptamer on the ability of JX-594 to infect a host cell as well as it's susceptibility to neutralizing antibodies (found in the serum of infected mice). All this will allow us to determine the suitability of each of the selected aptamers for *in vivo* studies.

Conclusion

In summary, the work performed in this project has identified two protocols that will allow for the selection of aptamers specific to a small viral target. When used serially, the protocols can diminish the number of unspecific aptamers present in a pool though they cannot fully eliminate them, when used either together or alone. Three candidate pools of aptamers binding to JX-594 have been identified and visualized by fluorescence shift in flow cytometry analysis and can be used for cloning and sequencing. Obtaining individual aptamer clones will facilitate investigation into their applications. Successful use of these aptamers will result in increased effectiveness of JX-594 as an oncolytic virus and will resolve some of the challenges currently associated with its production and use.

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