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Streptavidin-ZAP: Use of a Secondary Conjugate to Evaluate Potential Targeting Molecules

ABSTRACT

Targeted toxins -- targeting agents conjugated to saporin -- are widely used to eliminate specific cell populations both in vitro *and* in vivo*. For these molecules to be effective, it is vital that the targeting component of the conjugate specifically binds the cells of interest. A secondary conjugate, Streptavidin-ZAP, has been created by attaching the toxin saporin to streptavidin. The user can combine primary biotinylated material with Streptavidin-ZAP to quickly and economically screen potential targeting molecules for internalization and specificity. Once the appropriate targeting molecule is identified, a direct conjugation with saporin can be performed.*

INTRODUCTION

Targeted toxins, such as immunotoxins or ligand toxins, are effective tools for eliminating specific cells within a heterogeneous population. These molecules have been used to ablate cholinergic neurons in the basal forebrain,[1,2] neurokinin-1 receptor-expressing cells in the spinal cord,[3,4] macrophages in the intestine,[5] and many other cell types *in vivo* as well as *in vitro*. This technology allows scientists to determine cell function, to dissect complex pathways, and provides animal models of various diseases and syndromes that are cell receptor-specific.

Targeted toxins produced by Advanced Targeting Systems consist of the ribosome-inactivating protein, saporin[6] conjugated to a targeting molecule. The targeting molecule can be an antibody, peptide, protein, or any other molecule that recognizes a cell-surface marker. When the conjugate is administered, the targeting molecule directs the conjugate to the cells of interest. The conjugate is bound by the targeted cells, internalized, and saporin is released to inactivate the ribosomes. Cells not expressing the target do not bind or internalize the conjugate, and are not affected.

USE OF SECONDARY CONJUGATES

The efficacy of saporin has been well documented, however each prospective targeting molecule must be shown to work in a particular system. This may entail screening multiple candidates for internalization and specificity. Performance of antibodies as immunotoxins can easily be evaluated through the use of secondary immunotoxins, consisting of saporin conjugated to various secondary antibodies.[7] Streptavidin-ZAP is a tool that allows evaluation of targeted toxin efficacy using any molecule that can be biotinylated as a targeting agent. A diagram of a secondary conjugate is shown in Figure 1.

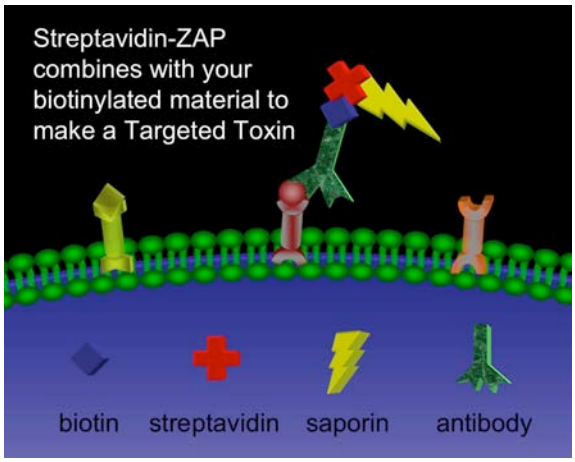

Figure 1

STREPTAVIDIN

The recombinant form of streptavidin has a molecular weight of 53 kDa. Streptavidin is a tetrameric protein; each subunit is able to bind one biotin molecule at a K_a of 10^{15} M^{-1}. The bond formation between streptavidin and biotin is rapid and essentially non-reversible, providing a simple and quick mechanism for creating targeted conjugates. The biggest hurdle to overcome in creating a targeted conjugate is nonspecific binding. If the conjugate binds to cells other than the population of interest the usefulness of the conjugate is compromised. Streptavidin has no carbohydrate group, and an isoelectric point (pI) of 5. These characteristics reduce non-specific binding, ensuring that cellular targeting is directed by the primary targeting agent, not by streptavidin. Avidin, the original protein associated with biotin, has a pI of 10, and is glycosylated. Streptavidin was chosen as a secondary conjugate component because it displays less nonspecific binding than avidin.

USE OF STREPTAVIDIN-ZAP

The first step toward using Streptavidin-ZAP is to biotinylate the targeting molecule. Advanced Targeting Systems offers biotinylation services if needed. The biotinylated molecule and Streptavidin-ZAP are then combined in equimolar concentrations. The streptavidin-biotin binding happens very quickly, little pre-incubation time is needed (30 minutes recommended) before the complex can be used. Once the complex is prepared it can be applied to the experimental system. One effective assay for screening targeting molecules is a cytotoxicity assay. Evidence of cell death *in vitro* can be demonstrated in 72 hours. Results of Streptavidin-ZAP use in a cytotoxicity assay are shown in Figure 2. In this assay, three reagents were added to cells: unconjugated saporin, a direct conjugate of IB4 to saporin (IB4-SAP), and Streptavidin-ZAP combined with biotinylated IB4. This assay compares the effectiveness of a direct conjugate with a secondary conjugate complex. As can be seen, this assay demonstrates that the results with the Streptavidin-ZAP/IB4 complex are highly reliable indicators of how effective a direct conjugate using IB4 would be. Thus, once you screen your potential targeting molecules with Streptavidin-ZAP, you can have a high level of confidence in having a direct conjugate produced.

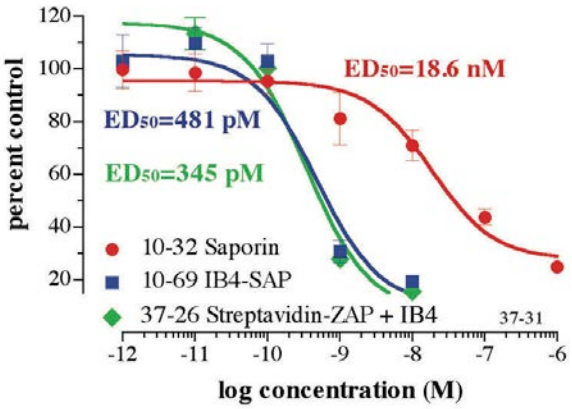

Figure 2.

KNRK cells are plated at 2500 cells/well and incubated overnight. Streptavidin-ZAP is premixed with Biotinylated-IB4 in equimolar concentrations. Saporin, IB4-SAP, and the Streptavidin-ZAP + Biotinylated IB4 mixture are then added in 10-µl volumes and the plates are incubated 72 hours. PMS/MTS developing reagent is added and the plates are incubated 15-30 minutes, then read at 490 nm.

TARGETED TOXINS ARE VERSATILE TOOLS

Targeted toxins eliminate specific cell populations; this function can be utilized in a variety of ways. In the context of a culture dish, cells that are considered contaminants can be removed without affecting the cells of interest. Streptavidin-ZAP can be used to establish whether targeting molecules are internalized. If the molecule is internalized, cell death will occur, an easily quantifiable event. Streptavidin-ZAP can also help determine whether cells are expressing a specific cell surface marker. Elimination of specific cell types can be very useful in experimental animals as well. By removing the cells of interest, intricate biological systems can be analyzed and individual cell function determined. Cells can also be targeted to establish an animal model of a particular disease or syndrome. For *in vivo* applications, it is recommended that the process be given two weeks to complete. That should allow sufficient time to remove all the antigens that might be used for evidence of cell loss.

SUMMARY

Targeted toxins are highly selective tools for removing specific cell populations. Secondary conjugates such as Streptavidin-ZAP are powerful tools for evaluating the targeting moiety of the conjugate. Streptavidin-ZAP can be tested *in vitro* and *in vivo*, but it is recommended that a direct conjugation be performed to provide a more stable product for extended *in vivo* use. Once the efficacy of a targeting molecule has been established, Advanced Targeting Systems can do a custom conjugation of the targeting molecule to saporin. Streptavidin-ZAP is extremely effective for the screening and selection of a targeting molecule. Once selected, a direct conjugation results in a more stable and active molecule that provides more consistent results, especially *in vivo*. For more information, references, or to request a quote for a custom conjugation, visit http://www.ATSbio.com.

REFERENCES

1. Leanza G, Nilsson OG, Wiley RG, Bjorklund A (1995) Selective lesioning of the basal forebrain cholinergic system by intraventricular 192-IgG-saporin: behavioural, biochemical and stereological studies in the rat. *Eur J Neurosci* 7:329-343.
2. Pappas BA, Payne KB, Fortin T, Sherren N (2005) Neonatal lesion of forebrain cholinergic neurons: Further characterization of behavioral effects and permanency. *Neuroscience* 133(2):485-492.
3. Mantyh PW, Rogers SD, Honore P, Allen BJ, Ghilardi JR, Li J, Daughters RS, Lappi DA, Wiley RG, Simone DA (1997) Inhibition of hyperalgesia by ablation of lamina I spinal neurons expressing the substance P receptor. *Science* 278:275-279.
4. Nichols ML, Allen BJ, Rogers SD, Ghilardi JR, Honore P, Luger NM, Finke MP, Li J, Lappi DA, Simone DA, Mantyh PW (1999) Transmission of chronic nociception by spinal neurons expressing the substance P receptor. *Science* 286:1558-1561.
5. Kanai T, Watanabe M, Okazawa A, Sato T, Yamazaki M, Okamoto S, Ishii H, Totsuka T, Iiyama R, Okamoto R, Ikeda M, Kurimoto M, Takeda K, Akira S, and Hibi T (2001) Macrophage-derived IL-18-mediated intestinal inflammation in the murine model of Crohn's disease. *Gastroenterol* 121:875-888.
6. Stirpe F, Barbieri L, Battelli MG, Soria M, Lappi DA (1992) Ribosome-inactivating proteins from plants: present status and future prospects. *Bio/Technol* 10:405-412.
7. Kohls MD, Lappi DA (2000) Mab-ZAP: A tool for evaluating antibody efficacy for use in an immunotoxin. *Biotechniques* 28(1): 162-165.