The future of antiviral immunotoxins

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RECEIVED OCTOBER 12, 2015; REVISED DECEMBER 2, 2015; ACCEPTED DECEMBER 3, 2015. DOI: 10.1189/jlb.2MR1015-468R

ABSTRACT

There is a constant need for new therapeutic interventions in a wide range of infectious diseases. Over the past few years, the immunotoxins have entered the stage as promising antiviral treatments. Immunotoxins have been extensively explored in cancer treatment and have achieved FDA approval in several cases. Indeed, the design of new anticancer immunotoxins is a rapidly developing field. However, at present, several immunotoxins have been developed targeting a variety of different viruses with high specificity and efficacy. Rather than blocking a viral or cellular pathway needed for virus replication and dissemination, immunotoxins exert their effect by killing and eradicating the pool of infected cells. By targeting a virus-encoded target molecule, it is possible to obtain superior selectivity and drastically limit the side effects, which is an immunotoxin-related challenge that has hindered the success of immunotoxins in cancer treatment. Therefore, it seems beneficial to use immunotoxins for the treatment of virus infections. One recent example showed that targeting of virus-encoded 7 transmembrane (7TM) receptors by immunotoxins could be a future strategy for designing ultraspecific antiviral treatment, ensuring efficient internalization and hence efficient eradication of the pool of infected cells, both in vitro and in vivo. In this review, we provide an overview of the mechanisms of action of immunotoxins and highlight the advantages of immunotoxins as future anti-viral therapies. J. Leukoc. Biol. 99: 911-925; 2016.

Introduction

Despite modern prevention and treatment strategies, viral infections and subsequent diseases remain a major health concern with devastating consequences associated with morbidity, mortality, and burdensome economic consequences. Currently, licensed drugs for the treatment of viral infections have several drawbacks, including toxicity and emergence of drug resistance. Hence, new and improved antiviral therapies with novel modes of action are urgently needed, and use of antiviral immunotoxins as “smart bombs” provide 1 such option.

Immunotoxins are fusion molecules that consist of a targeting molecule conjugated to a toxin molecule. The targeting molecule can be either antibody based, such as a mAb and a genetically engineered single-/double-chain antibody fragment or a receptor ligand, such as a growth factor or cytokine that targets specific cell surface receptors (Fig. 1) [1, 2]. The targeting molecule can be fused to a range of toxins, such as bacterial, plant, or fungal toxins, or to human apoptotic proteins. Although the toxin ensures efficient killing of the diseased or infected target cell, the targeting moiety ensures selectivity (i.e., that the killing moiety is directed to the diseased cells only). The selectivity must be accompanied by efficient internalization of the immunotoxin. Thus, selection of the appropriate immunotoxin target not only relies on the target expression profile, but must also take target internalization into account [3]. Furthermore, immunotoxins are highly immunogenic molecules and may rapidly elicit an immune response when administered to humans. Improvement in design of the immunotoxins is therefore warranted (e.g., identification and silencing of human T cell epitopes [4]) to overcome resistance and to achieve low immunogenicity for repeated treatment cycles.

Mostly, but not exclusively, immunotoxins are purpose-built to kill cancer cells. Thus, the identification of numerous unique cancer targets in recent years has led to the development of various malignancy-directed immunotoxins, with successful approval by the FDA (Table 1). The early immunotoxin denileukin difitox (Ontak, Eisai, Inc., Woodcliff Lake, Nj, USA) with the targeting molecule IL-2 fused to the C terminus of DT received accelerated approval for the treatment of CD-25-positive cutaneous T cell lymphoma in 1999 and received regular approval in 2008 [5–8]. A subclass of immunotoxins, known as immunoconjugates, contains an antibody for specific antigen targeting. The antibody is coupled to a variety of effector molecules by cleavable or uncleavable linkers that ensure optimal delivery of the effector molecule into the cell (Fig. 1).

Immuconjugates can be both highly specific and effective and with minimal toxicity if they are optimally designed. Therefore, certain immunoconjugates are considered among the most

Abbreviations: 7TM = 7 transmembrane, ACD = antibody drug conjugate, BLT = bone marrow-lymphoid axis, CD = Castleman’s disease, DT = diphteria toxin, Env = envelope, FDA = U.S. Food and Drug Administration, FTP = fusion toxin protein, gp = glycoprotein, GPCR = G protein-coupled receptor, HCMV = human CMV, HCV = hepatitis C virus, KSHV = Kaposi sarcoma-associated herpes virus, MCD = multicentric Castleman’s disease, (continued on next page)

1. Correspondence: Department of Neuroscience and Pharmacology, University of Copenhagen, Blegdamsvej 3, Copenhagen 2200, Denmark. E-mail: rosenkilde@sunz.ku.dk
promising anticancer therapies in the clinic [9]. There are 3 classes of immunoconjugates, based on the mechanism of action of the therapeutic agent conjugated to the antibody. The first class includes a pharmacological drug-like substance; members of this class are also known as ADCs. The second class consists of radionucleotides and the third class, of catalytic toxins (Fig. 1) [9]. The 2 most well-described immunoconjugates to date belong to class 1: brentuximab vedotin (targeting CD30) and trastuzumab emtansine (targeting HER2), approved by the FDA for the treatment of certain lymphomas and breast cancer, respectively [10, 11]. Radionucleotide immunoconjugates (class 2) (e.g., ibritumomab tiuxetan and iodine tositumomab) have shown clinical efficacy in the treatment of hematologic malignancies, but have failed in solid-tumor management [12–14]. Several immunoconjugates that involve a catalytic toxin (class 3) show promising results and are under clinical evaluation (phase I–III trials) and preclinical trials [9, 15]. Table 1 provides an overview of immunotoxins for treatment of cancers. Given the impressive progress in anticaner immunotoxin development, in particular regarding efficacy and safety, there is great potential of this technique for other clinical indications, such as infectious diseases, where pathogen-encoded targets provide superior specificity as compared to up-regulated endogenous target molecules in cancers. In the this review we focus on immunotoxins for the treatment of virus infections. At present, all antiviral immunotoxins are antibody based, yet 1 exemption recently entered the stage by presentation of the first antiviral immunotoxin targeting a virus-encoded 7TM GPCR denoted US28—not by an antibody, but by refinement of a chemotactic cytokine (chemokine) for optimal binding to US28 [16]. We discuss existing antiviral immunotoxins, novel viral targets for immunotoxins with main focus on virus-encoded 7TM GPCRs, and perspectives on the future of immunotoxin-based antiviral therapy.

IMMUNOTOXINS AS NOVEL ANTIVIRAL THERAPEUTICS

Today’s antiviral therapies intervene in the infection cycle of the virus by primarily targeting virus entry, intracellular virus replication, virus particle formation, and cell exit or by modulating cellular immune defense systems [17]. Immunotoxins as antiviral therapeutics are relatively underexploited, but are potentially useful, as these molecules can interfere with virus replication at the same time as killing virus-infected cells [16, 18]. The greatest advantage, in comparison with anticaner immunotoxins, is that the target molecule often is encoded by the virus, thereby preventing side effects encountered by killing of cells expressing low levels of the targeted human molecule (Table 2). In addition, interference with the protein synthesis within infected eukaryotic cells blocks virus dissemination and may eliminate the reservoirs in latently infected cells from which the virus reactivates [19].

Figure 1. Structure of immunotoxins and immunoconjugates. Immunotoxins are composed of a target molecule, that can be in the form of an antibody, a growth factor, or a cytokine that directs the immunotoxin to its specific molecular target, which is cell or virus derived. The target molecule and the therapeutic can be directly fused to each other or connected by a linker molecule. The therapeutic consists of a toxin molecule derived from plants, bacteria, or fungi or an apoptotic protein that will kill the targeted cell when internalized. Immunoconjugates always have an antibody as the target molecule, which is specific to cell- or virus-derived targets. The target molecule is combined to the therapeutic by a linker, and the therapeutic is a drug substance, a radio ligand, or a toxin.
### TABLE 1. Characteristics and developmental state of immunotoxins used for cancer treatment. The immunotoxins are listed in alphabetic order. Molecular targets of the immunotoxins are shown in bold. Immunotoxins described in the text are shown on grey background.

<table>
<thead>
<tr>
<th>Immunotoxin</th>
<th>Targeting moiety: molecular target</th>
<th>Toxin moiety</th>
<th>Type of cancer</th>
<th>Developmental stage</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>260F9 Mab-rRA</td>
<td>Anti-M	extsubscript{r} 55,000</td>
<td>RicA</td>
<td>Breast cancer</td>
<td>Terminated in phase I (1989)</td>
<td>[86, 87]</td>
</tr>
<tr>
<td>260F9 Mab-rRA</td>
<td>murine mAb (260F9): Mr 55,000 antigen</td>
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<td></td>
<td></td>
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<tr>
<td>454A12-rRA</td>
<td>Anti-human TFR mAb (454A12): TFR</td>
<td>RicA</td>
<td>Ventricular cerebrospinal fluid cancer</td>
<td>Phase I</td>
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<tr>
<td>A-dmDT390-bisFv</td>
<td>Anti-CD3 with addition of an extra sFv domain: CD3</td>
<td>DT</td>
<td>CTCL</td>
<td>Phase I</td>
<td>[90, 91]</td>
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<tr>
<td>Ado-trastuzumab emtansine (Kadcyla) (trastuzumab):</td>
<td>Anti-HER2 mAb (trastuzumab): ErbB2</td>
<td>DMI&quot;</td>
<td>Breast cancer</td>
<td>FDA approved</td>
<td>[11, 92–100]</td>
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<tr>
<td>Anti-B4-bRicin</td>
<td>Anti-CD19 mAb: CD19</td>
<td>Blocked Ricin</td>
<td>B-cell non–Hodgkin lymphoma</td>
<td>Terminated in phase III (2011)</td>
<td>[101–106]</td>
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<td>Anti-MY9-bRicin</td>
<td>Anti-CD33 mAb (MY9): CD33</td>
<td>RicB</td>
<td>AML</td>
<td>Terminated in phase I (1998)</td>
<td>[107]</td>
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<tr>
<td>B43-PAP</td>
<td>Anti-CD19 mAb (B43): CD19</td>
<td>PAP</td>
<td>ALL</td>
<td>Phase I</td>
<td>[108, 109]</td>
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<tr>
<td>Ber-H2-Sap6&quot;</td>
<td>Anti-CD30 mAb (Ber-H2): CD30</td>
<td>Saporin 6</td>
<td>Hodgkin disease</td>
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<td>[110–112]</td>
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<td>BL22 (RFB4(dsFv) PE38 or CAT-3888)</td>
<td>Disulfide-linked V	extsubscript{H} and V	extsubscript{L} chains of Anti-CD22 Fv fragment (RFB4): PE-38</td>
<td></td>
<td>B-cell malignancy</td>
<td>Phase II trials completed, superseded by moxetumomab pasidotox</td>
<td>[113–117]</td>
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<tr>
<td>Brentuximab vedotin (Adcetris)</td>
<td>Mouse-human chimeric IgG1 anti-CD30 mAb: CD30</td>
<td>MMAE&quot;</td>
<td>Hodgkin lymphoma; anaplastic large cell lymphoma</td>
<td>Accelerated FDA approval (10-19-2011)</td>
<td>[10, 118–123]</td>
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<tr>
<td>Cintredekin besudotox</td>
<td>IL-13: IL-13Ro2</td>
<td>PE truncated form, PE38QQR</td>
<td>Malignant glioma</td>
<td>Terminated in phase III (2010)</td>
<td>[124–127]</td>
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<tr>
<td>DA7</td>
<td>Mouse anti-CD7 human mAb: CD7</td>
<td>dgA</td>
<td>T-cell non-Hodgkin lymphoma</td>
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<td>[128, 129]</td>
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<tr>
<td>DAB&lt;sub&gt;486&lt;/sub&gt;IL-2&quot;</td>
<td>II.2: CD25</td>
<td>DAB&lt;sub&gt;486&lt;/sub&gt;</td>
<td>Hematologic cancer</td>
<td>Phase I/II</td>
<td>[130–134]</td>
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<tr>
<td>Denileukin diftitox (ONTAK)&quot;</td>
<td>II.2: CD25</td>
<td>DAB&lt;sub&gt;389&lt;/sub&gt;</td>
<td>CTCL</td>
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<td>DT390-GMCSF and DT388-GM-CSF</td>
<td>GM-CSF: GM-CSFR</td>
<td>DT390</td>
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<td>DT388-IL3 erb-38</td>
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<td>DT388</td>
<td>AML</td>
<td>Phase I/II&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>DAB&lt;sub&gt;486&lt;/sub&gt;IL-2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>IL-3: CD33 mAb (hP67.6): CD33</td>
<td>DAB&lt;sub&gt;486&lt;/sub&gt;</td>
<td>Breast cancer</td>
<td>Phase I</td>
<td>[147–149]</td>
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<tr>
<td>Gentuzumab ozogamicin (Mylotarg)</td>
<td>Anti-CD33 mAb (hP67.6): CD33</td>
<td>Calicheamicin</td>
<td>AML</td>
<td>FDA approved</td>
<td>[150]&lt;sup&gt;b&lt;/sup&gt;, [151–154]</td>
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<td>H65-RTA</td>
<td>Anti-CD5 mAb (H65): CD5</td>
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<td>Anti-CD19 mAb (HD37): CD19</td>
<td>dgA</td>
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<td>HUM-195/rGEL</td>
<td>Anti-CD33 humanized mAb (M195): CD33</td>
<td>rGel</td>
<td>Refractory myeloid leukemias</td>
<td>Phase I</td>
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<td>IL4-PE</td>
<td>Circulary permuted IL-4</td>
<td>PE38KDEL</td>
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<td>Ki-4.dgA</td>
<td>Anti-CD30 mAb: CD30</td>
<td>dgA</td>
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<td>B3(LysPE40) and LMB-1(B3(LysPE38))</td>
<td>Anti-Le	extsuperscript{Y} mAb (B3): LysPE-40 and LysPE-38</td>
<td>PE40</td>
<td>Breast cancer and colon cancer</td>
<td>Phase II</td>
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<tr>
<td>Anti-TAC(Fv)PE40 and LMB-2 (anti-TAC (scFv)PE38)</td>
<td>Anti-CD25 Fv portion (anti-tac): CD25</td>
<td>PE38KDEL</td>
<td>CD25	extsuperscript{V} T- and B- cell malignancies</td>
<td>Phase I, recruiting for a phase II study</td>
<td>[171–173]</td>
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<td>LMB-7 (B3(Fv)–PE38)</td>
<td>Anti-Le	extsuperscript{Y} mAb single chain Fv fragment [B3 (Fv)]: Lewis Y</td>
<td>PE38-KDEL</td>
<td>Neoplastic meningitis</td>
<td>Terminated in phase I (2000)</td>
<td>[174]</td>
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<tr>
<td>LMB-9 (B3(dsFv)–PE38)</td>
<td>Stable disulphide version of B3(Fv) [B3(dsFv)]: Lewis Y</td>
<td>PE-38</td>
<td>Bladder, lung, breast, GI tract and pancreatic cancers</td>
<td>Terminated in phase I (2003)</td>
<td>[175]</td>
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<tr>
<td>Moxetumomab pasudotox</td>
<td>RFB4 with point mutations Ser100Thr, Ser100His and Tyr100Gra in heavy-chain CDR3: CD22</td>
<td>PE38</td>
<td>B cell malignancies</td>
<td>Phase I, recruiting for a phase II study</td>
<td>[176, 177]</td>
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<td>MR1-1</td>
<td>Anti-EGFRvIII mAb scFv fragment: EGFR vIII</td>
<td>PE38KDEL</td>
<td>Glioblastoma multiforme</td>
<td>Phase I</td>
<td>[178]</td>
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<tr>
<td>N901-bR</td>
<td>Anti-CD56 mAb: CD56</td>
<td>Blocked ricin</td>
<td>Small cell lung cancer</td>
<td>Terminated in phase II (2002)</td>
<td>[179–181]</td>
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<td>OVB3-PE</td>
<td>Anti-ovarian cancer cell mAb (OVB-3): unknown antigen on ovarian cancer cells</td>
<td>PE</td>
<td>Ovarian cancer</td>
<td>Terminated in phase I (1991)</td>
<td>[182, 183]</td>
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<tr>
<td>RFB4-dgA</td>
<td>Anti-CD22 murine mAb (RFB): CD22</td>
<td>dgA</td>
<td>B-non Hodgkin lymphoma,</td>
<td>Phase I</td>
<td>[184–187]</td>
</tr>
<tr>
<td>RFB4-Fab-dgA</td>
<td>Fab	extsuperscript{V} fragment of RFB: CD22</td>
<td>dgA</td>
<td>B-non Hodgkin lymphoma</td>
<td>Phase I</td>
<td>[188, 189]</td>
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<td>RFT5-dgA	extsuperscript{C}</td>
<td>Anti-CD25 Ab: CD25</td>
<td>dgA</td>
<td>Hodgkin’s disease</td>
<td>Phase I/II</td>
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<td>ScFv(FRP5)-ETA</td>
<td>N-terminal single-chain Ab fragment (scFv) specific to ErbB2: ErbB2</td>
<td>PE</td>
<td>Breast, head, prostate and neck cancers.</td>
<td>Phase I</td>
<td>[195, 196]</td>
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<tr>
<td>SGN-10</td>
<td>Linked heavy- and light-chain variable regions of murine mAb BR96 (BR96 (scFv) ): Lewis Y</td>
<td>PE40</td>
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<td>Phase I</td>
<td>[197, 198]</td>
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<tr>
<td>SS1P</td>
<td>Anti-mesothelin single-chain Fv Ab: Mesothelin</td>
<td>PE-38</td>
<td>Mesothelioma, ovarian and pancreatic cancers</td>
<td>Phase I, recruiting for a phase II study</td>
<td>[199–202]</td>
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<tr>
<td>T101-ricin A chain</td>
<td>Anti-65 kD GP mAb (T101): 65 kD GP</td>
<td>RicA</td>
<td>CLL</td>
<td>Terminated in phase I (1988)</td>
<td>[203, 204]</td>
</tr>
<tr>
<td>TF-CRM107</td>
<td>Transferrin-C: TFR</td>
<td>DT (with mutations Leu390Phe Ser525Phe in the B chain)</td>
<td>Malignant glioma</td>
<td>Phase III completed in 2008</td>
<td>[205–208]</td>
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TABLE 1. (continued)

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<tr>
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<th>Developmental stage</th>
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<tbody>
<tr>
<td>TGα/3-TP40</td>
<td>TGF-α: EGFR</td>
<td>PE-40</td>
<td>Bladder cancer</td>
<td>Phase I</td>
<td>[209, 210]</td>
</tr>
<tr>
<td>TGα/3-TP38</td>
<td>TGF-α: EGFR</td>
<td>PE-38</td>
<td>Malignant glioma</td>
<td>Phase II</td>
<td>[211–214]</td>
</tr>
<tr>
<td>XomaZyme-791</td>
<td>Murine anti-colon carcinoma mAb (791T/36): M&lt;sub&gt;r&lt;/sub&gt; 72,000 antigens on colon carcinoma cells.</td>
<td>RicA</td>
<td>Colon cancer</td>
<td>Phase I</td>
<td>[215, 216]</td>
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<tr>
<td>XomaZyme-MEL (or XMMME-001-RTA)</td>
<td>Murine anti-melanoma mAb: melanoma antigens with a molecular weight of 220,000 and over 500,000</td>
<td>RicA</td>
<td>Melanoma</td>
<td>Phase II</td>
<td>[217–219]</td>
</tr>
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</table>

All, acute lymphoblastic leukemia; AML, acute myeloid leukemia; DAB, modified diptheria toxin; DAB389, the first 389 amino acids of DT; CIL, chronic lymphocytic leukemia; CTCL, cutaneous T-cell lymphoma; dgA, deglycosylated ricin-A-chain; DM1, derivative of maytansine 1; DT, diptheria toxin; EGFR, epidermal growth factor receptor; FCR-L-5, Fc receptor-like protein 5; GI, gastrointestinal; GM-CSFR, Granulocyte-macrophage colony-stimulating factor; HER, human epidermal growth factor receptor; kD, kilodalton; MC, mixed cryoglobulinemia; MAAE, Dolastatin 10 analog monomethyl auristatin e; MZ, marginal zone; PAP, pokeweed antiviral protein; PE38, genetically engineered deletion of amino acids 253-334 and 381-613 of PE; PE38KDEL, truncated form of PE where the amino acids KDEL is added in the C terminal region to increase toxicity; PE40, deletion of domain 1a of PE; rGEl, recombinant gelonin; RicA, ricin A chain; RicB, ricin B chain; TFR, transferrin receptor; VH, heavy chain domain of variable Ab region; VL, light chain domain of variable Ab region. Only immunotoxins based on non-classical (not bacterial, plant or fungal derived) toxins that are FDA approved are included in the table. Unpublished data. Immunotoxins tested as both cancer and viral treatment.

Antibody-based immunotoxins for virus targeting

Immunoconjugates have been developed against a variety of viruses, including small single-stranded RNA viruses, such as HIV, PCV, and HCV, and larger DNA viruses, such as herpesviruses. (Table 2 provides a comprehensive overview of antiviral immunotoxins.) In this review we focus on the most well-investigated immunotoxins against HIV and herpesviruses.

Antibody-based immunotoxins

Antibody-based immunotoxins were assessed as a monotherapy for HIV infection soon after the identification of HIV as the causative agent of AIDS [20–23], but were found to be ineffective [24–26]. At present, immunotoxins are under consideration for incorporation into HIV eradication protocols in combination therapy [18, 24, 27]. Today’s antiretroviral treatment provides a lifesaving and effective control of HIV infections [28], as it promotes dramatic reductions in viral load in blood and lymphoid tissues and is accompanied by significant recovery of CD4<sup>+</sup> T-lymphocyte counts and immune system function [29]. However, infected cell reservoirs and low-level replication of HIV persist during years of suppressive antiretroviral treatment, leading to viral rebound upon cessation of treatment [29]. Therefore, adherence to daily treatment is essential, but with the potential consequence that drug resistance of viral variants may emerge. Therefore, strategies for HIV eradication therapies have been pursued [18, 30, 31], and immunotoxins have been tested for HIV eradication in combination with an antiretroviral therapy. HIV cell entry is facilitated by the sequential interaction of the viral Env gp120 with CD4 on the surface of the cell [32]. Therefore, immunotoxins consisting of antibodies binding to gp120 linked to the truncated form of PE have been developed. The 3B3(Fv)-PE38 (hereafter, 3B3-PE) is an improved version of the CD4(178)-PE40 (hereafter, CD4-PE) immunotoxin [26, 29]. Both immunotoxins display highly potent and specific cytotoxic actions in vitro against replication of HIV in PBMCs and monocyte-derived macrophages [33, 34]. This effect is particularly noteworthy in view of the extremely low levels of surface Env expression in macrophages and the postulated role of macrophages in HIV persistence during antiretroviral treatment [34]. A thymus-liver SCID-hu mouse was used to examine the ability of both immunotoxins in combination with antiretroviral drugs to treat HIV infections in vivo. HIV tropism is confined to human tissue that is capable of supporting a productive infection of the virus. Thus, to establish HIV infection in a mouse model, SCID mice underwent implantation of human fetal thymus and liver under the mouse kidney capsule [35]. Both immunotoxins strikingly improved traditional anti-HIV treatment, but did not eradicate the infection. Recently, 3B3-PE has been tested further in a BLT humanized mouse model, which has been validated in the study of HIV persistence [18]. The process of bioengineering BLT mice results in systemic dissemination of human hematopoietic cells through the animal [36]. After effects of HIV infection on the BLT mouse human immune system were observed (e.g., CD4<sup>+</sup> T cell depletion and immune activation), antiretroviral therapy was established, and 3B3-PE was incorporated into the therapeutic regimen. 3B3-PE significantly improved the anti-HIV treatment, by profoundly depleting productively infected cells systemically, but did not eliminate the virus [18]. The tested immunotoxins can limit the size of the HIV reservoir, but cannot eradicate HIV in combination with the...
TABLE 2. Characteristics and developmental state of immunotoxins used for viral treatment. The immunotoxins are listed in alphabetic order. Molecular targets of the immunotoxins are shown in bold and further in italics if the target is virally expressed. Immunotoxins described in the text are shown on grey background.

<table>
<thead>
<tr>
<th>Immunotoxin</th>
<th>Targeting moiety: Molecular target (viral or human)</th>
<th>Toxin moiety</th>
<th>Type of infection</th>
<th>References</th>
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<tr>
<td>0.5β-RAC and 0.5β-PE</td>
<td>mAb 0.5β: gp120</td>
<td>RicA/PE</td>
<td>HIV</td>
<td>[220]</td>
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<tr>
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antiretroviral therapy. However, based on the results, alternative immunotoxins with different effector molecules and targeting moieties (Table 2) could be successful components of an effective HIV eradication therapy.

### Antibody-based immunotoxins targeting herpesviruses

Antibody-based immunotoxins targeting herpesviruses have shown promising results in several cases, as discussed in detail below.

#### Antibody-based immunotoxins against KSHV.

KSHV (also known as HHV-8) is an oncogenic virus that has been characterized as the etiologic agent in the onset and development of KS, PEL, and MCD [37, 38]. Like other herpesviruses, KSHV possesses 2 distinct transcriptional programs: latency and lytic replication [39]. In the latent phase, viral genomes are maintained silent within the infected cells, and in the lytic phase, all lytic viral genes are expressed in a tightly regulated cascade, after which the progeny virus is assembled and released from the infected cell. Two immunotoxins have been developed for selective killing of lytically infected cells: YC15-PE38 and 2014-PE38 [40, 41]. They both consist of a single chain Fv mAb targeting KSHV lytic glycoproteins linked to the truncated form of PE (PE38). The targeted glycoproteins are expressed on KSHV particles to facilitate virus entry, and consequently, the spread of the infection in infected cells in vitro [46]. R33ExoA is the first immunotoxin designed for targeting a virus at a mucosal site of infection, but to be considered for anti-HSV-2 therapy, its efficacy under exceptional conditions in vivo, such as low vaginal pH, has to be proven.

#### Antibody-based immunotoxins against HCMV.

HMCV can cause severe diseases in immunocompromised persons and establishes life-long infection in the human body [47]. Because the virus is highly species specific, it is not easily studied in animal models (except humanized mouse models). Instead, its murine counterpart MCMV is often used as a surrogate model for the study of virus replication and pathogenesis in vivo [48, 49]. Immunotoxins specific for cells infected with HCMV and MCMV have been constructed by linking polyclonal anti-HCMV and -MCMV IgG to gelonin, a type I ribosome-inactivating protein. Both immunotoxins failed to obtain sufficient anti-HCMV/anti-MCMV activities in vitro [50], indicating that other immunotoxins with improved antiviral activities are needed for in vivo studies. Examples of these are mAbs and pAbs conjugated to a deglycosylated ricin-A chain for MCMV targeting. These
immunotoxins were more efficient than the gellonin-based anti-MCMV immunotoxin and were therefore evaluated in vivo in MCMV-infected SCID mice [51]. Unfortunately, they were both inadequate in their effects on survival [52] and their overall effect was weak in comparison to that of ganciclovir in mice. Further work is needed to determine whether more potent and efficient antibody-based immunotoxins against CMV can be found.

CHEMOKINE-BASED IMMUNOTOXINS FOR HCMV TARGETING VIA US28

The first chemokine-based immunotoxin targeting a viral GPCR has been reported recently [16]. The targeting molecule was not antibody-based, but instead was created by a series of modifications in the cognate chemokine-ligand for the HCMV-expressed chemokine receptor US28. GPCRs constitute the biggest protein family in the human genome and are targets for ~50% of all currently marketed drugs [53]. Based on their 7TM α-helices, they are also known as 7TM receptors. The chemokine receptors and ligands constitute important parts of a very complex system that controls leukocyte movements during homeostasis and inflammation. Herpesvirus genomes have evolved an extraordinary repertoire of tools designed to ensure successful infectivity and propagation. A large fraction of these tools involves virus-encoded 7TM receptors and ligands, most of which have high genetic and functional homology to either chemokines or chemokine receptors. Thus, HCMV devotes a significant part of its genome to immune modulatory gene homologs, as the virus-encoded GPCRs UL33, UL78, US27, and US28, with US28 being a functional chemokine receptor [34]. Several pharmacological and cellular properties of US28 suggest that this vGPCR would be suitable for targeting of HCMV in an immunotoxin-based strategy. First, although US28 binds a broad spectrum of chemokines as part of its proposed immune evasive function as a chemokine scavenger, it shows high selectivity and enhanced binding for the CX3C chemokine CX3CL1 [55]. Because CX3CL1 binds only 1 human chemokine receptor, CX3CR1, the potential of unwanted off-target effects of a CX3CL1-based immunotoxin strategy is decreased. Second, CX3CL1 consists of a chemokine domain, a mucin stalk, and a transmembrane domain. These structural characteristics suggest that this chemokine can sustain high-affinity binding to US28 when the C-terminally attached mucin-like stalk is replaced by another protein (e.g., the cytotoxic domains of PE). Third, detailed studies indicate that most of the US28 receptors localize away from the cell surface in endosomes [56, 57]. This distribution results from rapid, constitutive, and ligand-independent receptor internalization [58], a feature well-suited for efficient intracellular delivery of immunotoxins. Based on the molecular characteristics of US28 and its defined ligand profile, an immunotoxin was designed consisting of the chemokine domain CX3CL1 and the translocation and cytotoxicity domains of PE (Fig. 2). It was given the name CX3CL1-FTP to highlight that the target moiety was based on a chemokine, not an antibody [16]. CX3CL1-FTP binds to US28 with higher affinity, kills US28-expressing cells with higher potency, and more efficiently controls virus replication and release of virus particles than does ganciclovir. Moreover, CX3CL1-FTP is capable of controlling virus replication of a ganciclovir-resistant HCMV strain—a highly important property for treatment of patients who have an increasing clinical challenge of infection with ganciclovir-resistant HCMV strains. As CX3CL1-FTP also killed cells expressing the human chemokine receptor CX3CR1 (albeit much less efficiently), a rational design strategy was applied to further enhance the selectivity of the immunotoxin molecule toward US28 [16]. A single-point mutation (Phe49 to Ala) in CX3CL1 retained high-affinity binding to US28 and reduced affinity to CX3CR1, providing a favorable selectivity profile toward US28. The selectivity-optimized immunotoxin F49A-FTP exhibited far superior control of HCMV infection in vivo in the SCID-hu mice model compared with ganciclovir [16, 59]. The efficacy, combined with the rational design of a selective chemokine as the target molecule, demonstrates the high therapeutic potential of this drug candidate. Many questions clearly remain, including the capacity for development of F49A-FTP-resistance in vivo and toxicity and the immunogenicity associated with F49A-FTP.

7TM receptors as potential antiviral immunotoxin targets

As evident from the presentation of F49A-FTP, the immunotoxin drug candidate for the treatment of HCMV, rationally designed immunotoxins targeting virus-expressed receptors may provide promising drug targets, not only for anti-HCMV therapy, but also other virus-encoded 7TM receptors [60–63].

The EBV-encoded BILF1 receptor. EBV encodes 1 constitutively active tumorigenic receptor, EBV-BILF1 [61, 62, 64]. It has been suggested that this 7TM receptor is involved in the pathogenesis of EBV-associated malignancies, presumably in a signaling-dependent manner. The molecular properties of EBV-BILF1 indicate that this receptor is a promising drug target and is suitable for immunotoxin targeting. Cell surface expression of EBV-BILF1 can be detected during the lytic phase of infection, and even low receptor expression levels were detectable in latency [65]—an important feature for targeting EBV-associated cancers as only a few viral gene products are expressed. Moreover the receptor is constitutively internalized into the cell [66], which is important for efficient toxin delivery into cancer cells. However, as EBV-BILF1 is an orphan receptor, a ligand has to be identified for the immunotoxin design.

The KSHV-encoded chemokine receptor ORF74. This broad-spectrum chemokine receptor seems highly suitable for immunotoxin targeting. In itself, ORF74 can induce the onset of Kaposi’s sarcoma–like lesions through the activation of a complex network of signaling pathways that involve the autocrine and paracrine activation of proliferative, proinflammatory, and angiogenic pathways. Targeting ORF74-mediated signaling by inhibiting individual pathways has been shown to have insufficient therapeutic potential to cure several cancers [67]. In contrast, using immunotoxins designed to target KSHV-infected cells through ORF74 seems a valid approach to efficiently kill KSHV-infected cells; hence, the abrogation of oncogenic signaling events. ORF74 has a defined chemokine ligand profile [68–70] and is internalized in response to human CXCCL1 and -8 [71]. Thus, a rational chemokine-based immunotoxin strategy in homology to the strategy outlined above for US28 and CX3CL1 could be applied.
In addition to the viral 7TM receptors, several viruses exploit host-encoded receptors by different means: 1) by up-regulating them in the same manner that EBV up-regulates EBI2 [72], 2) by using them as cell entry cofactors [73–76], or 3) by encoding ligands (agonists and antagonists) for endogenous receptors, as vMIP1–3 is encoded for KSHV [77–79]. In theory, immunotoxins could also target these 3 methods. For the up-regulated receptors, such as EBI2 (also known as GPR183), it has been suggested that the up-regulation improves virus replication, and that it could be important for antiviral immune defense [80, 81]. The role of EBI2 in the EBV life cycle is still uncertain [81]. If EBV benefits from high EBI2 expression, an immunotoxin targeting EBI2 could provide an efficient and specific way to inhibit EBV-associated diseases. A possible drawback of this approach would be cytotoxic side effects in all EBI2-expressing cells (B cells, T cells, macrophages, dendritic cells, and many others) [82]—effects that could be reduced by lowering the dose. However the reduction, in turn, could lead to inefficient immunotoxin targeting [83, 84].

**FUTURE DIRECTIONS AND LIMITATIONS OF ANTIVIRAL IMMUNOTOXINS**

For future antiviral immunotoxins to be successful, they must be efficient, with low toxicity. This efficiency can be obtained by using the following criteria: 1) The target of the immunotoxins must be highly disease-specific to limit side effects. Immunotoxins targeting the viral genome or virus-encoded proteins expressed on the surface of virions and infected cells are efficient targets, as these molecules are not normally expressed on human cells. 2) The target must be expressed in both the latent and the lytic phase of the virus cycle, to completely eradicate the virus infection. Indeed, although effective killing of lytically infected cells is not enough to eradicate viruses that also have a latent stage, it is sufficient in most cases to treat acute infection. 3) The target expressed on the surface of infected cells must have a rapid internalizing capability for the immunotoxin to be efficiently delivered to the intracellular environment. 4) The immunotoxin must bypass the host’s immune system as generation of neutralizing antibodies to the immunotoxin prevents continued treatment and retreatment. It is especially the toxin moiety of the immunotoxin that can be immunogenic, as this is often derived from bacteria or plant (nonhuman) sources. To circumvent this problem, patients could be treated with immunosuppressive drugs to prevent or delay the production of neutralizing antibodies [85].

**CONCLUSION**

Current development of antiviral immunotoxins is an emerging field with constantly improved and innovative methods for successful application. Research has developed combination therapies with immunotoxins that are beneficial in viral treatment, which further highlights the promise of successful application of antiviral immunotoxins.

**AUTHORSHIP**

K.S., M.H.J., T.N.K. and M.M.R. contributed to the development of ideas, literature research, and writing and editing the text, table, and figures.

**ACKNOWLEDGMENTS**

The authors thank Line Barington for critical reading of the manuscript and helpful comments.

**DISCLOSURE**

The authors declare no conflicts of interest.
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The future of antiviral immunotoxins
Katja Spiess, Mette Høy Jakobsen, Thomas N. Kledal, et al.

Access the most recent version at doi:10.1189/jlb.2MR1015-468R

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