Immunotoxins and Other Conjugates: Pre-clinical Studies

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Abstract: A wide variety of conjugates containing RIPs, of either chemical or recombinant type, have been made and tested against dangerous cells *in vitro* and in animal models. Many of these pre-clinical studies will be reviewed here dividing them on the basis of the target cell and the surface molecule specifically recognized.

Keywords: Ribosome-inactivating Proteins, Toxins, Conjugates, Immunotoxins, Targeting, Immunotherapy

INTRODUCTION

Toxin conjugates consist of a targeting polypeptide covalently linked to a toxic protein. Monoclonal antibodies and antibody fragments have been the most frequently used targeting proteins, but also hormones, growth factors, antigens, cytokines and other receptor-binding substances have been commonly employed. While the term "immunotoxins" generally refers to a toxin targeted by either an intact antibody, a Fab fragment or a Fv fragment, toxins targeted by other ligands are more commonly referred to as "chimeric toxins" or simply "conjugates". Some conjugates were obtained by introducing a chemical linkage between the carrier and the toxin, and are sometimes referred to as "chemical conjugates" or "chemical immunotoxins". Otherwise, when the linkage is a peptide bond produced by genetic engineering, the conjugates are referred to as "recombinant conjugates" or "fusion toxins" or, when the carrier is a Fv peptide, "recombinant immunotoxins".

A wide variety of immunotoxins, of either chemical or recombinant type, have been made and tested against dangerous cells *in vitro* and in animal models. Many of these pre-clinical studies will be reviewed here dividing them on the basis of the target cell and the surface molecule specifically recognised. In each case, the possible practical application of the immunotoxin, either experimental or clinical, will be specified. The review will be divided into immunotoxins targeting haematological cells, growth factor receptors, transferrin receptor, solid tumours, HIV-infected cells, and acetylcholine receptor.

1. IMMUNOTOXINS TARGETING HAEMATOLOGICAL CELLS

Many immunotoxins have been developed against haematological cells, with the aim to elaborate both anti-neoplastic and immunosuppressive therapies. Haematological cells have been extensively studied and targeted with immunotoxins because (i) they have well known and characterised surface molecules against which a panel of mAbs is available, (ii) fresh cells may be easily tested for immunotoxin activity, and, (iii) these cells are easy to target *in vivo*.

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1.1. Immunotoxins Targeting CD2

CD2 is a 50 kDa glycoprotein expressed on the surface of T cells, NK cells, granulocytes and on subsets of monocytes and B cells. Anti-CD2 ITs have been devised for the therapy of $CD2^+$ lymphomas or leukaemias, and for T-dependent immune disorders, such as transplanted organ rejection and graft-versus-host disease.

Three ricin A chain-containing ITs, each recognising different epitopes on the CD2 molecule, were obtained by using RFT11, 35.1 and 9.6 mAbs. RFT11-IT, which recognises an epitope closer to the cell membrane, was 100-1000-fold more effective in killing normal and malignant T cells than the other two ITs. Moreover RFT11-RTA, was retained for a longer period of time inside the cells and was more slowly degraded than the less effective 35.1-IT, which was rapidly transported to lysosomes, digested, and expelled. This demonstrates that the epitope recognised by an IT may have a significant impact on the insertion into cell membrane, translocation to the cytosol, and cytotoxicity of the IT [1].

Three immunotoxins containing OKT11 (α-CD2), SOT3 (α-CD3), and SOT1a (α-CD5) mAbs were obtained by covalent coupling to the type 1 RIP saporin. The α-CD2 immunotoxin OKT11-saporin was 10-fold less effective than the other two ITs, with an IC_{50} on DNA synthesis of 60 nM. However, OKT11-saporin, similarly to SOT3- and SOT1a-containing ITs, was able to accomplish T lymphocyte killing after less than 10 min exposure in the absence of adjuvant molecules [2]. α-CD2 ITs composed of saporin linked to OKT11 and 7A10C9 mAbs were very active in inhibiting protein synthesis in IL-2 activated lymphocytes and lymphoma D430B and Raji cell lines, with IC_{50}s ranging from less than 5×10^{-13} to 3×10^{-12} M. Bone marrow purging from contaminating lymphoma cells by means of $CD34^+$ cell purification and subsequent OKT11-saporin treatment resulted in >5 logs of lymphoma cells killing, without any significant reduction of the clonogeneic properties of stem cells [3].

In a comparative study, different CD antigens were targeted by specific mAbs linked to saporin. An α-CD2 mAb was effective when targeted by an indirect α-mouse IgG IT (IC_{50} $<10^{-11}$ M), but was much less efficient as a component of a direct α-CD2 IT (IC_{50} $<10^{-8}$ M). Moreover, the latter IT resulted 1-2 logs less active on PHA-stimulated peripheral blood lymphocytes than α-CD5, α-CD45 and α-CD3 ITs [4].

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Various α-CD2 mAbs (GT2, OKT11, 8E5B3, 8G5B12, 7A10C9) were used by Tazzari *et al.* [5] to produce different saporin-containing immunotoxins. These α-CD2-saporin conjugates inhibited protein synthesis by a neoplastic $CD2^+$ cell line (SKW-3) and by an interleukin 2-dependent polyclonal $CD2^+$ lymphoid cell culture (T lymphoblasts), with IC_{50}s ranging from 10^{-13} to 10^{-11} M. Similar results were obtained on PHA driven lymphoid cultures and on mixed lymphocyte culture activated lymphocytes. Moreover, an α-CD2-ricin A chain IT (8E5B3-RTA) resulted less effective than the corresponding saporin-containing IT on PHA-stimulated lymphocytes, with an IC_{50} of 10^{-9} M versus 10^{-12} M.

To evaluate the possibility of an *in vivo* therapy with IT constituted of a murine monoclonal antibody and a type 1 RIP, Reimann *et al.* [6] administered to macaque monkeys an anti-IL-2R mAb and a CD2-specific mAb both linked to the RIP gelonin. They observed the development of high-titer antibody responses to gelonin and to mouse Ig of different idiotypes and isotypes. Sera from treated monkeys prevented the *in vitro* cytotoxic effect of immunotoxins, by blocking both the mouse Ig and the RIP. Serum from a monkey infused with a CD2-specific mAb blocked the *in vitro* cytotoxicity of CD2-specific ITs containing isotypically different α-CD2 mAbs and of an IT containing a mAb of unrelated specificity. These results indicate that the humoral immune response to mAbs and toxins might limit or preclude the *in vivo* use of immunotoxins.

1.2. Immunotoxins Targeting CD3

SOT3-saporin α-CD3 IT rapidly (10 min) reacted with target cells, reaching an IC_{50} value on DNA synthesis of 4.5 nM, and its cytotoxicity was not potentiated by the lysosomotropic amines ammonium chloride, chloroquine, and amantadine [2]. Bolognesi *et al.* [4] comparing immunotoxins against different CD antigens, namely CD2, CD3, CD5 and CD45 found that an IT composed of an α-mouse IgG $F(ab')_2$ linked to saporin was equally toxic to human lymphocytes pretreated with anti-CD2, -CD3, and -CD45 mAbs, with IC_{50}s < 10^{-11} M, whereas a direct α-CD3 IT showed the highest specific toxicity on these cells, with IC_{50} 2.1×10^{-10} M. In the same study lymphocytes pretreated with α-CD3 were then incubated with α-mouse IgG ITs containing eight different type 1 RIPs and RTA. Those containing PAP-S and saporin resulted the most active with IC_{50}s 2.6 and 5.5×10^{-12} M, respectively.

The cytotoxicity of the WT32-RTA α-CD3 IT to the T-ALL Jurkat cell line was compared with its rate of internalization and intracellular degradation. The results showed that cytotoxicity occurred in two phases, with a rapid internalization of initially bound IT followed by a continuous but slower uptake, possibly due to re-expression of the CD3 antigen. Exocytosis of intracellularly degraded molecules became measurable after 1 to 2 h and increased during the following 24 h. NH_4Cl and monensin enhanced the cytotoxicity of WT32-ricin A chain strongly reducing the degradation of internalized IT [7].

Anti-CD3-RTA and anti-CD3$F(ab')_2$-RTA promoted engraftment in C57BL/6 mice of T-cell-depleted marrow deriving from haemopoietic histocompatibility (Hh1) Ag positive (BALB/c mice) or negative (DBA/1 mice) allogeneic donors, with a long lived positive effects on engraftment. These results provided definitive data that $CD3^+$ cells participate in the rejection of either $Hh1^+$ or Hh1null T cell-depleted allografts [8]. $F(ab')_2$, lacking the Fc fragment, has the advantage of not activating T cells. An anti-CD3 $F(ab')_2$-dgA was used to treat graft-versus-host disease induced across major or minor histocompatibility barrier in mice. This IT was potent and selective in inhibiting T-cell mitogenesis *in vitro* (>95% inhibition) and *in vivo* in mice receiving BM transplants (80% of T cells depletion). A 5-day course of 30 μg/d i.p. of IT, beginning 7 days after GVHD induction significantly prolonged survival of mice (MST greater than 80 days versus 20-30 days of untreated animals). In this model (B10.BR mice) the IT half-life was about 9 hours [9].

An immunotoxin prepared by conjugating the α-CD3 mAb 64.1 to dgA was used to prevent the development of human B lymphomas in SCID mice reconstituted with normal PBL from EBV^+ human donors. The incidence of the disease was greatly reduced by either a combination of *in vitro* treatment of PBL followed by one *in vivo* treatment of the xenografted mice with 64.1-dgA IT or by *in vivo* repeated treatments. This IT did not have any non-specific cytotoxic effects on already established EBV^+ tumours and was proposed as a simple method to avoid lymphoproliferative disease when injecting blood-containing tissues into SCID mice [10].

Administration of two-three doses of an α-CD3-RTA IT directed against the CD3 ε-chain induced a complete, long-lived, and selective protection against the onset of experimental autoimmune diabetes in mice. A second RTA-containing IT directed against Ly1, the murine homologue of human CD5, also protected in a dose-dependent manner from diabetes, but was less effective than the α-CD3-RTA IT. These studies demonstrate that an α-CD3 IT may be useful *in vivo* for the treatment of diabetes and perhaps other T-cell-mediated autoimmune diseases [11].

1.3. Immunotoxins Targeting CD4 and/or CD8

α-CD4 and α-CD8 immunotoxins containing intact ricin, in the presence of 0.1 M lactose, were able to reduce PBMC proliferation by 40 and 70%, respectively, with an increment in the response by more than 95% with the use of a combination of the two ITs [12]. Similar results were obtained with other mAbs and RIPs. TEC-T4 mAb linked to saporin, inhibited PHA-induced PBMC proliferation with an IC_{50} of about 1 nM, that decreased to 0.1 nM by using a combination of αCD4/αCD8 mAbs and an α-mouse IgG IT [13]. G17-2 (α-CD4) and G10.1 (α-CD8) mAbs linked to ricin both inhibited clonal proliferation of target T-cell with a maximum of about 3 logs at 5 nM concentration [14]. M-T151 mAb linked to RTA inhibited CEM cells protein synthesis, with an IC_{50} of 4.6 nM. The corresponding ITs obtained by linking blocked or unblocked intact ricin to the same antibody showed IC_{50} values of 20-30 pM; the cytotoxicity of both ITs being reduced by 3- to 4-fold in the presence of lactose [15]. These ITs affected only minimally the clonogeneic properties of bone marrow stem cells. CD4 was utilised also as a target for anti-HIV immunotherapy.

1.4. Immunotoxins Targeting CD5

CD5 (gp67) is a developmental marker of T lymphocytes and is also present on the surface of B cells subsets. Anti-CD5 ITs have been devised as a tool for the therapy of T-cell lymphomas or leukaemias and $CD5^+$ B-chronic lymphocytic leukaemia, and of T-dependent immune disorders, such as transplanted organ rejection and graft-versus-host disease.

T101 mAb has been probably the most widely used α-CD5 antibody. In a first research, Manske *et al.* [16] found that T101-RTA modulated CD5 more efficiently than T101-ricin and T101 alone. They found also that modulation, which is undesirable in monoclonal antibody therapy, is advantageous in the therapeutic use of IT, and that the presence of the toxin prevented antibody shedding from PBMC. T101-ricin was found to inactivate protein synthesis in leukaemic cell lines, in the presence of lactate, more efficiently than α-CD2, α-CD7 and α-gp95,170 [17]. T101-RTA was found to be less effective than T101-ricin, however, it does not require the presence of lactose to avoid unspecific toxicity. T101-RTA cell toxicity was enhanced by monensin [16,18], amantadine [19], ammonium chloride and, moderately, by verapamil [20]. Further experiments demonstrated that the rate-limiting step in the toxicity difference between T101-ricin and T101-RTA was the rate of transfer to lysosomes that was significantly slowed by ricin B-chain, and that trafficking through the Golgi is important for IT cell toxicity [18]. However, other scientists found that lysosomal degradation and cleavage of disulfide-linked conjugates is a quantitatively minor phenomenon as compared with the localisation of internalized anti-CD5 ITs in an endosomo-Golgi compartment [21]. T101-ricin was used *in vivo* in nude mice s.c. injected with the acute lymphoblastic leukaemia T-cell line CEM, to produce subcutaneous solid tumours. A combination regimen of IT and mafosfamid induced a 72-100% reduction in tumour volume, 3 to 4 days after intratumour injection treatment, but tumours relapsed within 5 to 13 days. Persistent tumour regression was observed with successive injections of the combined therapy, with 86% of mice surviving more than 30 days post-treatment and 5/7 mice with no detectable tumour on day 30 [22]. An α-CD5 radioimmunotoxin was produced by labelling with ^{125}I an immunoconjugate consisting of ricin and the T101 mAb. This conjugate provided information related to biodistribution and pharmacokinetics. Four days following intratumoural injection, more than 125-fold greater radioactivity was found in CEM tumours, as compared to normal tissues. The mean blood half-life of the ^{125}I-IT was 25.7 h [23]. Preclinical studies showed that cyclosporin A and its non-immunosuppressive analogue, SDZ PSC 833, enhanced the cytotoxic activity of T101-RTA (by 101- and 105-fold, respectively) and of the more potent T101 $F(ab')_2$-RTA (by 9- and 8-fold, respectively) on CEM III, a human lymphoblastic T-cell line. A significant increase in T101-RTA-mediated cytotoxicity, ranging from 31% to 60%, was induced, *in vitro*, by clinically achievable concentrations of cyclosporin A (3.1-4.8 ng/mL) [24].

H65 mAb has been another murine α-CD5 antibody extensively studied for its targeting properties. H65-RTA was specifically cytotoxic for PBMC after both prolonged (90 h) and short (2 h) incubation time, in the absence of potentiators. In contrast, most T cell lines were more sensitive to IT in the presence of potentiators, and IT cytotoxicity was much less enhanced by prolonging the exposure time [25]. Chimeric human-mouse H65 $F(ab')_2$ and Fab' were produced by genetic engineering in *E. coli*. These antibody fragments, once purified, were conjugated to RTA either by direct coupling to the unique free cysteine of the toxin (Fab') or after derivatisation with 2-iminothiolane ($F(ab')_2$). Both these ITs efficiently killed a $CD5^+$ T-cell line (HSB2) and human peripheral blood T cells. The IC_{50} values on PBMC [^3H]thymidine uptake were: H65-RTA 0.28 nM, $F(ab')_2$-RTA 0.089 nM, Fab'-RTA 2.2 nM, as toxin [26]. The same recombinant antibody fragments, conjugated to RTA or to recombinant gelonin (rGel), were tested in a human peripheral blood lymphocyte-reconstituted SCID mouse model. cH65 $F(ab')_2$-rGEL and cH65 Fab'-rGEL conjugates were essentially equally effective at depleting human T-cells from SCID mouse. cH65 Fab'-RTA was not as effective as cH65 $F(ab')_2$-RTA or H65-RTA in depleting human T-cells, both *in vivo*, in SCID mice, and *in vitro*, in human PBMC. In the latter system, cH65 Fab'-RTA was 17-fold less potent than cH65 $F(ab')_2$-RTA and approximately 50-fold less potent than the rGEL conjugates [27]. H65 mAb and its Fab and $F(ab')_2$ fragments were conjugated also to a family of GEL analogs, each with a single unpaired cysteine residue. Several rGEL analogs formed immunoconjugates that were up to 6-fold more cytotoxic to antigen-bearing cells than those made with linker-modified rGEL, whereas other analogs were less potent. In the rat, the *in vivo* clearance rates of whole antibody conjugates correlated with their relative disulfide bond stability *in vitro*. Fab conjugates did not show this correlation, suggesting that the clearance of Fab conjugates depends more on the removal of intact immunoconjugate from serum than on deconjugation mechanism [28]. The LD_{50} of H65-RTA was estimated to be between 60 and 62.5 mg/kg in the rat. Repeated doses of H65-RTA administered to rats and cynomolgus monkeys, produced peripheral edema, decreased body weight, decreased body temperature (monkey only) and a general inflammatory reaction. Inflammation, haemorrhage, and/or edema were evident in a variety of tissues. Monkeys developed much more severe toxicity than rats. However, toxicity was reversible in both animals. Since H65 antibody does not cross-react with T cells from either rats or cynomolgus monkeys, the toxicity observed in these studies was probably due to a series of acute to subacute inflammatory reactions caused by the RTA moiety [29].

Parameters affecting tumour-specific delivery of various anti-CD5-ricin A chain immunotoxins were studied *in vivo* in BALB/c nude mice bearing human ascitic Ichikawa tumour cells. In this study, it was found that tumour cell localisation of whole IgG/RTA IT molecules after i.v. administration was very low (1,600 molecules/cell) if compared to i.p. administration (25,000 molecules/cell). Moreover, it was found that contemporaneous Fc removal and partial deglycosylation of RTA increased IT tumour localisation by 5.5-fold, by both reducing plasma clearance and increasing extravasation of the conjugate [30]. The cytotoxicity of the same ITs increased when monensin conjugated to human serum albumin was contemporaneously

injected i.p., whereas no changes were observed after i.v. injection [31].

The exponential or stationary growth phase was found to affect the sensitivity of Jurkat leukaemic cells to the α-CD5 ST.1-RTA IT. The time required to kill one log of target cells was 70 h in proliferating and 12 h in stationary cells. This difference was greatly diminished by the addition of the IT enhancer monensin (4.9 h versus 3.5 h in proliferating and stationary cells, respectively). Binding and internalization studies revealed that the higher sensitivity of stationary cells to ST.1-RTA treatment was not due to an increased uptake or to a faster internalization kinetics, but rather to a different intracellular routing of IT molecules [32]. Afterwards, long exposures of Jurkat leukaemia cells to this IT were found to select a resistant tumour-cell population, still sensitive to RTA, but with a transient modulation of CD5 antigens (1.1% of recycled CD5 molecules/h) [33].

Recently, an α-CD5/ricin IT was found to effectively decrease the proliferative capability of one-way MLC-stimulated cord blood and peripheral blood T cells, at concentrations (10^{-9}-10^{-8} M) which did not affect the growth of haematopoietic progenitor cells [34].

Several other RIPs, other than ricin or its A chain, were conjugated with α-CD5 mAbs.

The mistletoe lectin-I A-chain (MLA) was conjugated with an α-CD5 mAb, resulting 80-fold more toxic to CD5-bearing target cells than the corresponding conjugate with ricin A-chain [35].

An immunotoxin containing SOT1a (α-CD5) mAb was obtained by covalent coupling to the type 1 RIP saporin. SOT1a-saporin IT showed a rapid reaction with target cells (10 min) and an IC_{50} on DNA synthesis of 1.4 nM. The lysosomotropic amine amantadine only moderately potentiates the cytotoxicity of this IT (IC_{50} 0.36 nM) [2]. The same authors realised an OKT1 mAb-saporin IT. This IT had a very fast kinetics. T-lymphocyte killing was achieved by a five-minute exposure of the target cells. The IC_{50} on T-lymphocyte DNA synthesis was 0.32 nM. The potency of OKT1-SAP was slightly enhanced by amantadine (IC_{50} 0.08 nM), but not by ammonium chloride or chloroquine. Following a single i.v. injection of non-toxic dosages (0.16-1.3 mg/kg) of OKT1-SAP in cynomolgus monkeys, an initial rapid decline ($t_{1/2}\alpha$ = 1.0-4.1 hours) was followed by a long-lasting slower decrease ($t_{1/2}\beta$= 11.6 to 20.6 hours) of plasma concentrations [36]. OKT1-SAP was capable of eliminating *in vitro* more than 95% of clonable T-lymphocytes infiltrating an irreversibly rejected renal allograft, despite their resistance to previous aggressive immunosuppression (cyclosporine plus steroids, high-dose steroids, intravenous OKT3 antibody, and local irradiation) [37]. This IT was also tested on fresh B-chronic lymphocytic leukaemia (B-CLL) cells from 31 consecutive patients. In 90% of cases, OKT1-SAP specifically suppressed B-CLL cell proliferation in a dose-related manner, with IC_{50}s 4.0-6.8 nM [38].

An α-CD5 immunotoxin containing the type 1 RIP momordin showed a very high potency on PBMC *in vitro* (IC_{50} = 1-10 pM) and inhibited the development of Jurkat T cell leukaemia in nu/nu mice by 80% [39]. On Jurkat cells this IT showed an IC_{50} of 41-53 pM after a 72 h incubation, whereas 1 h was sufficient for 6 pM immunotoxin to inhibit 50% of PBMC protein synthesis. Studies on the internalization pathway indicate that the low cytotoxic activity of IT on tumour cells, detected in the short-term assay, was due to inefficient delivery to its cytoplasmatic target, while a longer exposure of the cells to the immunotoxin promotes adequate intracellular distribution [40].

1.5. Immunotoxins Targeting CD7

CD7 is a 40 kDa determinant expressed by most T-ALL and a majority of normal mature peripheral T cells. This antigen was first targeted using the 3A1 antibody conjugated to PAP, which was reported to eliminate 4.8 logs of leukaemic cells from normal bone marrow, at an IT concentration (3 μg/ml, about 10^{-8} M) only slightly toxic to stem cells (13% CFU-GEMM decreasing). This IT completely inhibited PHA-induced mitogenic response of PBMC, thus, resulting potentially useful for the treatment of GVHD in allogeneic bone marrow transplantation as well [41]. Further experiments showed that by using 3A1-PAP in combination with 2'deoxycoformycin and deoxyadenosine it was possible to reach a 6 logs of HSB-2 T lymphoma cells elimination, although a significant increment in bone marrow toxicity was evidenced [42].

With the perspective of bone marrow purging in autologous transplantation, the cytotoxic properties of an α-CD7 WT1-RTA IT were investigated on CD7-expressing malignant T cells. WT1-RTA cytotoxicity was dependent on antigen density and was enhanced considerably in the presence of 6 mM ammonium chloride. In a clonogenic assay more than 6 logs of CEM cells were eliminated after incubation with 10^{-8} M IT. At that concentration no adverse effects on progenitor cells were observed. CD7 antigen recycling on the cell membrane enables continuous uptake of IT resulting in a time-related increased malignant T cells elimination [43].

In a murine model of human leukaemia the treatment with the 4A2-RTA30 α-CD7 IT resulted in a 100- to 200-fold specific depletion of CEM cells from the spleen and the bone marrow [44].

Another animal model (MT-ALL cell line transplanted into SCID mice) was established to test the DA7 IT, constructed by chemically linking the mouse IgG2b α-CD7 3A1E mAb to the deglycosylated ricin A-chain (dgA). DA7 selectively inhibited protein synthesis and led to greater than 5 logs kill of clonogenic MT-ALL cells, *in vitro*. This IT administered to mice at 10 μg/mouse/day for 5 consecutive days, starting 8 days after i.v. inoculation of leukaemia, gave a significantly longer-term survival, over 348 days, with 82% of treated animals free from disease, as compared to control mice which were all dead by day 70. SCID mice given i.p. injections of leukaemic cells developed an i.p. tumour mass and a disseminated leukaemia in haematopoietic and non-haematopoietic organs. This leukaemia was fatal in 100% and killed the animals in 68-95 days. SCID mice given i.p. injections of MT-ALL completely responded to therapy with DA7, resulting in survival of 100% of the animals at 216 days [45].

SCID mice transplanted with MOLT-3 human T-lineage acute lymphoblastic leukaemia cells were used to test an α-CD7 IT (G3.7-PAP). SCID mice were treated i.p. with IT at doses of 5 μg/mouse/day on days 1-3. Of nine treated mice, four died in 54-149 days and five remained alive for > 172 days; some of them developing disseminated therapy-refractory leukaemic blasts [46].

HB2 has been one of the most used mAbs to produce α-CD7 ITs. Saporin was effectively delivered to the T-ALL cell line HSB-2 by a $F(ab'\gamma)_2$ BsAb, specific for the RIP and the CD7 antigen, with IC_{50} 0.23 nM and a rate of protein synthesis inactivation very similar to that described for conventional immunotoxins (t_{10} of 46 h at 1 nM saporin concentration and 226 h at 0.1 nM) [47]. The cytotoxic performance of two HB2-derived BsAbs, also reacting with saporin, was investigated *in vitro*. On HSB-2 cells the α-CD7 BsAb HB2 × DB7-18 gave a 435-fold increase in saporin toxicity [48]. However, in comparative experiments, the immunotoxin HB2-SAP resulted six times more effective than HB2 × DB7-18 and 98 times more effective than the quadroma produced BsAb Q1.1 [49].

A HB2-SAP IT specifically inhibited HSB-2 cell line protein synthesis, *in vitro*, with an IC_{50} of 4.5 pM, and significantly prolonged the survival of SCID mice injected with HSB-2 cells. This therapeutic effect was seen with a single injection of 10 μg of IT given at day +7 [50], and was even more evident when IT was administered as three daily injections of 10 μg on day +7, +8, and +9 [51]. Further experiments revealed that host-mediated ADCC contributes to the *in vivo* therapeutic efficacy of HB2-SAP IT, as demonstrated by the reduced activity of an IT constructed with the HB2 $F(ab')_2$ fragment, which is incapable of recruiting NK cells [52], and the reduced activity of HB2-SAP in NOD/SCID mice, which have reduced cytolytic NK activity [53]. *In vivo* comparison of the same immunotoxin constructed with either a hindered (HB2-SMPT-SAP) or non-hindered (HB2-SPDP-SAP) disulphide bond [54], and containing one or two saporin moieties [55] failed to reveal significant difference in pharmacokinetic [54] and therapeutic effect [54,55].

The α-CD7 TXU-PAP IT tested in a T-ALL/SCID mouse model, caused a marked improvement in the number of leukaemia-free surviving animals. Whereas 100% of control mice died of disseminated human leukaemia within 80 days (median survival, 37 days), 80±13% of SCID mice treated with 15 μg and 100% of mice treated with 30 μg of TXU-PAP remained alive and free of leukaemia for >120 days. In cynomolgus monkeys, TXU-PAP was eliminated with an half-life of 8.1-8.7 h. The monkeys treated with TXU-PAP at doses of 0.05 mg/kg/day × 5 days and 0.10 mg/kg/day × 5 days tolerated well the therapy, without any significant clinical compromise or side effects [56].

1.6. Immunotoxins Targeting CD19

CD19 is a 95 kDa glycoprotein that functions as a response regulator, which modulates B-cell differentiation. It is expressed on the B lymphocyte lineage from the beginning of B-cell commitment to plasma cell differentiation, and is also present on B-cell lymphomas and leukaemia.

A first α-CD19 IT was obtained by chemically linking the murine IgG1 B43 mAb to the type 1 RIP PAP. This conjugate, *in vitro*, eliminated greater than 99.96% of B-ALL blast progenitors freshly obtained from patients [57], whereas in the presence of chloroquine killed 5.8 logs of Burkitt's lymphoma cells [58] and in combination with mafosfamide (ASTA Z 7557) produced nearly 7 logs of elimination of B-ALL cells from normal human bone marrow [59].

A B43-PAP IT showed antitumour activity in several murine models. In SCID mice transplanted with 1×10^6 CALLA positive human pre-B acute lymphoblastic leukaemia cells (NALM-6-UM1), a dose of 10 μg/mouse × 3 days of IT delayed from 38 to 74 days the development of leukaemia: a result consistent with ≥6 logs kill of clonogenic NALM-6-UM1 cells in 64% of SCID mice. The probability of long-term event-free survival (EFS) was 65±10% for mice treated with 15 μg B43-PAP and 60±11% for mice treated with 30 μg B43-PAP, with a median survival time of greater than 7 months [60]. The antitumour activity of B43-PAP was also tested on $CD19^+$ BCL-1 murine B-cell leukaemia, transplanted in BALB/c mice [61], and RS4;11 human acute leukaemia cell line, transplanted in SCID mice [62]. In both model, B43-PAP reduced the burden of disseminated leukaemia and improved the survival of treated animals. Its effect was also evident against radiation-resistant primary B-cell precursor leukaemia cells, and conferred extended survival to SCID mice xenografted with the same cells [63]. Intrathecal therapy with B43-PAP immunotoxin at non-toxic dose levels, significantly improved survival of SCID mice with a CNS xenografted leukaemia (NALM-6 human cells) and was superior to intrathecal methotrexate therapy [64].

The combination of B43-PAP with cyclophosphamide (CPA), markedly improved the EFS of SCID mice challenged with 5×10^6 NALM-6-UM1 cells. The probability of EFS at 6 months resulted 50±16 % for mice treated with 1 mg CPA plus 15 μg B43-PAP and 70±15 % for mice treated with 2 mg CPA plus 15 or 30 μg B43-PAP. The probability of long-term EFS was 14±7 % for mice treated with 30 μg B43-PAP and 0 % for mice treated with 1 mg CPA, but 40±16 % for mice treated with 30 micrograms B43-PAP plus 1 mg CPA [65]. Similarly, a B43-PAP/CPA combination therapy of SCID mice challenged with 5×10^7 RS4;11 leukaemia cells, markedly prolonged the median survival time from 41 days (control animals) to > 150 days, with a probability of EFS at 5 months of 90±10%. Mice treated with either B43-PAP or CPA alone showed median survival values of 79 and 80 days, respectively [66]. B43-PAP plus cytosine arabinoside combination showed potent anti-leukaemic activity against human B-cell precursor leukaemia in SCID mice and led to 100% long-term EFS from an otherwise invariably fatal leukaemia. None of the other treatment protocols tested, including combinations of B43-PAP with carmustine, doxorubicin, or etoposide, proved more effective than B43-PAP alone [67]. Similar results were obtained with B43-PAP/temozolomide combination, which, in the same NALM-6/SCID mice model, was not significantly more effective than B43-PAP alone [68].

Antileukaemic activity of B43-PAP was compared to that of vincristine (VCR), methylprednisolone (PDN) and L-

asparaginase (L-ASP) as single agents, as well as in a 3-drug combination regimen ("VPL") using the NALM-6/SCID mouse model. In these experiments, B43-PAP resulted more active than VCR, PDN, or L-ASP and the two-drug combinations VCR + B43-PAP, PDN + B43-PAP, or L-ASP + B43-PAP were not significantly more active than B43-PAP. When mice were challenged with 5×10^6 NALM-6 cells, all of them rapidly died of disseminated leukaemia with a median EFS of 37±3 days. The 3-drug combination "VPL" (median EFS = 75±23 days) was slightly less active than B43-PAP (median EFS = 84±19 days), but, notably, the combination of "VPL" with B43-PAP resulted in 100% survival [69]. Similar results were obtained in SCID mice challenged with 1×10^6 NALM-6 cells (MST = 41.2 days), in which the treatment with the thymidylate synthase inhibitor ZD1694 (TOMUDEX) improved the median survival to 69.2 days, B43-PAP treatment resulted in 51.0% long-term EFS, with a median survival of 187.5 days, and the combination treatment resulted in 100% long-term leukaemia-free survival [70].

Preclinical pharmacokinetics, biodistribution and toxicity studies of B43-PAP were carried out in different animal models. B43-PAP composed of two PAP molecules linked to each mAb molecule was found to have greater chemical stability, higher systemic exposure levels and a more effective anti-leukaemic activity in a B-ALL/SCID mouse model, as compared to the 1 to 1 conjugate [71]. B43-PAP administered to BALB/c mice caused fatal cardiac and renal toxicities, in addition to multifocal skeletal myofiber necrosis, with abnormal gait and lethargy. This IT related toxicity was markedly reduced by parenteral administration of methylprednisolone, pentoxyphylline, or dopamine [72]. In cynomolgus monkeys B43-PAP, administered as a single i.v. bolus dose, showed a slow blood clearance (1-2 ml/h/kg), without significant equilibration with the extravascular compartment. Multiple i.v. IT administration was well tolerated, with no signs of toxicity, at total dose levels ranging from 0.007 to 0.7 mg/kg. At 0.35 mg/kg a transient episode of a mild capillary leak was observed, whilst B43-PAP total dose levels of 3.5 and 7.0 mg/kg caused a severe and dose-limiting renal tubular toxicity [73].

Another α-CD19 mAb, HD37, along with its $F(ab')_2$, or Fab' fragments, was conjugated with RTA or dgA. These ITs were specifically toxic to normal B-cells and to most of the neoplastic B-cell lines tested. ITs containing intact HD37 mAb and native or deglycosylated RTA chain reduced protein synthesis in Daudi cells by 50% at 1.5×10^{-10} M. ITs constructed from the Fab' fragments were 10- to 20-fold less potent than their intact antibody counterparts [74]. This anti-tumour activity was at least in part due to IT binding to CD19, since HD37 mAb was found to have antiproliferative activity on three $CD19^+$ Burkitt's lymphoma cell lines (Daudi, Raji, and Namalwa) (IC_{50} = 5.2-9.8×10^{-7} M) [75].

HD37-dgA was tested in SCID mice with disseminated human Daudi lymphoma, where it produced about 2 logs of tumour cells killing [76]. Moreover, this IT was able to induce a statistically significant reduction in the number of viable cells, *in vitro*, in primary culture of patient-derived pre-B ALL cells [77].

HD37 mAb conjugated to saporin, produced an IT that killed more than two logs of clonogenic B-chronic lymphocytic leukaemia cells from patients, with a two-hour incubation at a concentration non- toxic to non target cells [78].

To target CD19 with a derivative of ricin, which would have the translocating function of the ricin B-chain, another α-CD19 mAb, anti-B4, was conjugated to whole ricin blocked for the galactose-binding sites of the B-chain (blocked-ricin, bR) [79]. Blocked ricin consists of two species, one with two covalently attached ligands and one with three covalently attached ligands. Cytotoxicity studies revealed that anti-B4-bR made with blocked ricin containing two ligands was capable of depleting 5 logs of human target cells, while anti-B4-bR comprised of blocked ricin with three ligands could deplete only one log of cells [80]. These results were in agreement with the later findings that the residual binding activity of bR to cell surface molecules plays an important role in triggering cell calcium mobilisation and in increasing the internalization rate of anti-B4-bR IT [81]. Anti-B4-bR, at concentrations ≥ 5×10^{-9} M, could eliminate more than 3 logs of $CD19^+$ NALM-6 (human non-T and non-B ALL) or Namalwa (human B-cell lymphoma) cells, in a 20-fold excess of normal irradiated bone marrow, after 5 h of incubation [82]. This IT, at a dosage of 0.1 mg/kg i.v. administered for 5 days, was effective in killing up to 3 logs of the same tumour cells transplanted in SCID mice [83].

Preclinical pharmacokinetics and toxicity studies of anti-B4-bR were carried out in SCID mice, and cynomolgus and rhesus monkeys. Damage to the reticuloendothelial system was described for SCID mice i.v. treated with 0.1 mg/kg for 5 days, with IT blood concentrations ranging from 150 to 800 ng/ml [83]. In monkeys, which lack the CD19 epitope recognised by the anti-B4 mAb, a $t_{1/2}\alpha$ of 1.4-2.0 h and a $t_{1/2}\beta$ of about 14 h was reported, after a bolus injection. After 5 or 7-days treatment, minimal to moderate clinical signs of hepatic toxicity were described [84].

Anti-B4-bR was tested in combination with doxorubicin or etoposide on Namalwa cells and the P-glycoprotein-expressing cell line Namalwa/mdr-1. In both cases the combined treatment produced supra-additive effects, *in vitro*. *In vivo*, treatment with 5 daily bolus injections of anti-B4-bR (50 μg/kg) followed by treatment with doxorubicin (3 mg/kg) or etoposide (15 mg/kg) significantly increased the life span of Namalwa/mdr-1/SCID mice by 129% and 115%, respectively. After treatment with anti-B4-bR, the multi-drug resistant Namalwa/mdr-1 population expressed lower levels of P-glycoprotein, rendering these cells more sensitive to doxorubicin and etoposide and accounting for the synergistic action of the drug combinations [85]. Better results were obtained with multidrug regimens. Treatment with five daily bolus i.v. injections of anti-B4-bR followed by CHOE (cyclophosphamide, vincristine, doxorubicin, and etoposide) increased the life span of Namalwa/mdr-1-bearing SCID mice by 173%, and 20% of the animals were completely cured. Combination therapy with anti-B4-bR and CCE (cyclophosphamide, cisplatin, and etoposide) produced long-term complete cure in 50% of the tumour-burdened mice [86].

Immunotoxin BU12-saporin was constructed by covalent coupling the single chain RIP saporin and the α-CD19 mAb BU12. This IT was selectively cytotoxic *in vitro* in a dose-

dependent manner for the $CD19^+$ B-ALL cell line NALM-6, but exhibited no toxicity for the $CD19^-$ T-ALL cell line HSB-2. The survival of SCID mice challenged with NALM-6 cells was significantly prolonged, compared with sham-treated control animals, by a course of therapy with BU12-saporin but not with an irrelevant α-CD7 IT [87]. Similar results were obtained with SCID mice challenged with the $CD19^+$ $CD38^+$ human Burkitt's lymphoma cell line Ramos, treated with 3 doses of BU12-saporin IT, starting at day 7 from tumour cell injection [88].

1.7. Immunotoxins Targeting CD22

CD22 is a 135 kDa B-cell restricted sialoglycoprotein, which has a role as a component of the B-cell activation complex and as an adhesion molecule. CD22 is expressed on the B-cell surface only at mature stages of differentiation and is also expressed by the majority of B-cell malignancies.

In a first comparative work, four α-CD22 mAb (RFB4, HD6, UV22-I and UV22-2), or their Fab' fragments were conjugated to RTA, and the toxicity of the resulting ITs was tested on Daudi (Burkitt's lymphoma), NALM-6 (B-ALL) and ARH-77 (myeloma) cell lines. The IgG-RFB4-RTA killed 50% of Daudi cells at a concentration of 1.2×10^{-12} M and 50% of NALM-6 and ARH-77 cells at concentrations of $1.5\text{-}2.1\times10^{-11}$ M, and it was 10-30 times more toxic to Daudi cells than were the other three α-CD22 IgG-RTA ITs. As frequently observed, Fab'-containing ITs were 2-5 times less toxic to target cells than their IgG-containing counterparts [89]. In SCID mice challenged with Daudi cells (MST 45.9±4.3 days), whole IgG- or Fab'-containing RFB4-dgA ITs, administered at day +1, extended the mean survival time to 87.2±18.9 days and 57.9±3.8 days, respectively [90]. RFB4-dgA also induced a statistically significant reduction in the number of viable cells, in patient-derived pre-B leukaemic cells maintained *in vitro* [77], and significantly improved survival of SCID mice transplanted with NALM-6-UM1 human pre-B ALL cell line [91]. To further improve the anti-tumour efficacy of RFB4/dgA IT, a combination with a CD19-targeted radioimmunotherapy (^{131}I-HD37 mAb) was evaluated in SCID mice with a disseminated human lymphoma. When used alone, both ^{131}I-HD37 and IT therapy were safe and effective, but not curative. When the two therapies were combined, efficacy and toxicity became dependent on the temporal order of administration. Thus, when ^{131}I-HD37 was administered after IT therapy, the regimen was curative. In contrast, when ^{131}I-HD37 was administered before RFB4/dgA, the combination induced a life-threatening pulmonary vascular leak [92].

HD6 α-CD22 mAb, along with its $F(ab')_2$, or Fab' fragments, was coupled to RTA or dgA, obtaining ITs specifically toxic to normal B-cells and to most of the neoplastic B-cell lines tested. ITs containing intact HD6 mAb and native or deglycosylated RTA chain reduced protein synthesis in Daudi cells by 50% at 1.2×10^{-11} M. ITs constructed from the Fab' fragment were 10- to 20-fold less potent than their intact antibody counterparts [74].

Another α-CD22 IT was realised by using the IgG1 mAb CLB-B-ly/1 chemically linked to the recombinant RTA (rRTA). This IT was found to be highly cytotoxic, *in vitro*, to the B-cell line Ramos, with an IC_{50} of 3.5×10^{-11} M and the killing of 2.7-logs of cells. *In vivo*, a 5-day i.v. IT treatment of nude mice s.c. xenografted with Ramos cells, resulted in a transient tumour reduction. Anti-tumour activity was more pronounced in a disseminated tumour model in SCID mice. IT treatment (i.v.) 7 days after i.v. inoculation with Ramos cells resulted in complete cure of all mice. No relevant signs of systemic toxicity were observed at therapeutic concentrations of 5×30-5×60 μg/mouse. Blood clearance was characterised by a $t_{1/2}\alpha$ of 12.1 h and a $t_{1/2}\beta$ of 53.6 h. The LD_{50} in SCID mice after i.v. administration was 0.626 mg IT per mouse, whilst no death was observed after i.p. injection of 1 mg IT per mouse [93]. The IT anti-tumour activity diminished when the IT was administered after increasing the time lag following inoculation of tumour cells. Delaying IT administration resulted in growth of solid tumours. This implies that cells migrate to sanctuaries protected from the IT and indicates that the anti-tumour activity is strongly influenced by the accessibility of the IT to the target cells [94]. The cytotoxicity of this α-CD22 IT, *in vitro*, was strongly increased by the carboxylic ionophores monensin (81 to 117-fold) and nigericin (318 to 382-fold) and by the combination of the lysosomotropic amines chloroquine and NH_4Cl (107 to 145-fold). Cytotoxicity was not or slightly influenced by brefeldin A, all-trans retinoic acid (ATRA), verapamil, perhexiline maleate and protease inhibitors [95]. None of the enhancers were active in the presence of human serum or could reach *in vivo* effective/non-toxic concentrations [94,95].

HD6 and HD39 mAbs conjugated to saporin gave ITs recognising two different epitopes on the CD22 molecule. These ITs inhibited DNA synthesis and protein synthesis in target B lymphoma cells with a dose-related effect, in short incubation times and in the absence of potentiators. In a clonogenic assay >2 logs of malignant B cells were eliminated with two-hours incubation at concentrations nontoxic to CD22 non target cells [78].

Saporin was effectively delivered to malignant B-cell by four different α-CD22/α-saporin $F(ab')_2$ BsAbs, but not by α-CD19, α-CD37 and α-Ig. Each α-CD22 BsAb increased saporin toxicity up to 1000-fold, reaching IC_{50} values of $1.5\text{-}6.0\times10^{-10}$ M for both Daudi and Raji target cells. Pairs of α-CD22 BsAb, which recognised different non-blocking epitopes on the saporin molecule were able to bind saporin more avidly to the target cell and, as a consequence, increased saporin cytotoxicity lowering the IC_{50} to 2.0×10^{-11} M [96]. Similar results were obtained with α-CD22/α-gelonin $F(ab')_2$ BsAbs, which increased gelonin cytotoxicity to levels equivalent to that reached by saporin in the same targeting system [97]. A CD22/saporin BsAb was used as an experimental tool to demonstrate that most peripheral blood myeloma plasma cell precursors are mature or later B cells presenting membrane CD22 [98]. The exceptional ability to translocate the immunocomplex and accumulate saporin inside the cell of the CD22 antigen was further demonstrated to be due to an internalization motif within the cytoplasmic tail of the molecule [99].

Three different type 1 RIPs (momordin, PAP-S and saporin) were linked to the α-CD22 mAb OM124. These ITs were probably the most effective in inhibiting protein synthesis by various $CD22^+$ target cell lines (Daudi, EHM, BJAB, Raji and BM21), with IC_{50}s ranging from $<5\times10^{-15}$

(all ITs on EHM cells) to 7.6 $\times 10^{-11}$ M (momordin- and PAP-S-ITs on BJAB cells), and IC_{90} ranging from 5×10^{-14} to 5×10^{-10} M. The protein synthesis inhibition was found to correlate with apoptotic cell death. The immunotoxins, alone or in combination, significantly extended the survival time of SCID mice bearing transplanted Daudi cells. A combination of the three ITs injected i.p. to a total dose of 50% the LD_{50} extended the survival time of mice challenged with 5×10^6 Daudi from 39±5 (controls) to 73±10 days. A combination of cyclophosphamide (30 mg/kg, at days +1, +2) and OM124/saporin (0.17 mg/kg at days +3, +6, +9) was particularly effective in SCID mice transplanted with a lower number of cells (3×10^6), with a 66% of animals tumour-free after 220 days. In the same experiment, OM124-saporin alone (0.17 mg/kg at days +1, +4, +7), gave a complete remission in 33% of the animals [100].

1.8. Other Conjugates Targeting Plasma Cells

ITs specific for human myeloma cells were obtained by covalently linking the 8A mAb, recognising a plasma cell-associated antigen, to saporin [101], momordin [102] and momorcochin [103], and the α-plasma cell 62B1 mAb to saporin [101]. 8A-saporin was toxic for target cell lines, showing IC_{50}s on clonogenic growth of 1.0×10^{-12} M (Raji cells) and 4.0×10^{-11} M (U266). 8A-momordin was toxic for Raji and U266 cell lines, with IC_{50}s on clonogenic growth of 5.8×10^{-10} M and 5.2×10^{-10} M, respectively. 8A-momorcochin resulted less toxic for target cells, with IC_{50}s on Raji protein synthesis of 5.2×10^{-9} M and on clonogenic growth of 1.01×10^{-9} M. 62B1-saporin showed an IC_{50} of 4.0×10^{-13} M on the clonogenic growth of U266 cells.

An immunotoxin directed toward the intracellular adhesion molecule-1 (ICAM-1, CD54) was prepared by coupling the UV3 mAb to deglycosylated ricin A-chain. The epitope on ICAM-1 recognised by this antibody is strongly expressed on human myeloma cells, pre-B leukaemia cells and Burkitt's lymphoma cell lines, but is not expressed by normal PMNC, PBMC and resting B cells. In protein synthesis inhibition assays UV3-dgA was highly cytotoxic to the human myeloma cell lines HS-SULTAN (IC_{50} = 1.0×10^{-11} M) and ARH-77 (IC_{50} = 9.0×10^{-11} M) [104].

The lectin peanut agglutinin (PNA) was found to bind to multiple myeloma cells and a PNA-RTA was toxic toward Raji target cells, showing IC_{50} in the 10^{-7}-10^{-8} ng/ml range [105].

Experimental Heymann's Nephritis was induced in rats by immunizing them with purified renal brush border antigen (gp330). Twelve days after the antigenic challenge the administration of a gp330-gelonin conjugate resulted in a strong reduction of circulating α-gp330 antibodies and in the absence of proteinuria, indicating the possibility of a toxin-antigen therapy for this autoimmune renal disease [106].

Two mAbs, B-B2 and B-B4, reacting with human plasma cells CD138 antigen were conjugated to saporin and tested on a multiple myeloma cell line (RPMI8226) and on freshly isolated multiple myeloma bone marrow mononuclear cells. B-B2-saporin and B-B4-saporin resulted toxic for target cells in both tests. On RPMI8226 cell protein synthesis IC_{50} values were 10^{-9} M (B-B2-SAP) and 10^{-10} M (B-B4-SAP) [107]. The B-B4-saporin IT resulted less toxic to drug-resistant RPMI8226 with respect to drug-sensitive RPMI8226 in a clonogenic assay, with 2.5 logs kill and 5 logs kill, respectively. However, on malignant-myeloma cells from patients no difference was seen between drug-sensitive and drug-resistant cells treated with the same IT [108].

1.9. Immunotoxins Targeting CD25

CD25 (α-IL-2α receptor) is an antigen involved in allograft rejection, GVHD, autoimmune disorders and certain neoplastic diseases.

The α-CD25 IT B-B10-saporin inhibited ^{3}H-TdR incorporation in lymphocytes when stimulated with PHA (IC_{50} 3.12×10^{-12} M) or with a MLR (IC_{50} 3.23×10^{-13} M), and in an alloreactive T cell clone (IC_{50} 9×10^{-12} M) [109].

The IT RFT5-dgA inhibited the growth of H-RS cells at a concentration of 7×10^{-12} M and destroyed about 60% of solid Hodgkin's tumours of 0.5 cm diameter in nude mice, inducing complete remissions in 95% of SCID mice with disseminated tumours when administered one day after tumour challenge [110]. SCID mice treated 12 days after tumour challenge had lower remission rates (46%), suggesting that the antitumour effect of the immunotoxin depends on the number of tumour cells present [111].

The success of allogeneic bone marrow transplantation depends on an efficient prevention of GVHD and on the protection of patients from relapse and infections. The IT RFT5-dgA specifically allodepleted T cells and killed autologous CMV-infected fibroblasts and autologous EBV-B-lymphoblastoid cell lines [112].

Conjugates of α-CD25 mAbs with MLA (viscumin A chain) and RTA were highly cytotoxic on target cells. The α-CD25-MLA had an IC_{50} 15-fold greater than the α-CD25/RTA [113].

1.10. Immunotoxins Targeting CD30

CD30 is a member of the TNF receptor family. It was originally described as a marker of Hodgkin and Reed-Sternberg cells in Hodgkin's lymphoma. Expression of CD30 is mostly restricted to virus-infected lymphocytes, neoplasms of lymphoid origin and a subset of activated T cells, which produce Th2-type cytokines.

Five α-CD30 mAbs and two Fab' were linked to RTA and evaluated for their potential for the treatment of Hodgkin's disease. HRS-3-RTA and Ber-H2-RTA showed the highest cytotoxicity on L540 cells and the lowest cross-reactivity with normal human tissues [114]. In mice with solid s.c. L540 tumours of 60-80 mm^3 size, a single i.v. injection of a dose corresponding to 40% of the LD_{50} of Ber-H2-RTA, HRS-3-RTA, or another anti-Reed-Sternberg IT, Irac-RTA, induced lasting complete remissions in 38, 44, and 50% of animals, respectively. Irac-RTA treatment induced complete remissions in 100% of mice with small tumours (10-20 mm^3) but only 13% of mice with larger tumours (400-600 mm^3) [115].

Ber-H2 was conjugated with several type 1 RIPs, namely saporin, momordin, PAP-S and dianthin, always giving ITs

specifically inhibiting protein synthesis by Hodgkin-derived cell lines, with IC_{50}s ranging from 10^{-11} to 5×10^{-14} M [116,117,118].

Because of a transient hepatotoxicity observed in patients treated with Ber-H2/saporin, its accumulation by rat liver cells was studied. Adherent *in vitro* cultured non-parenchymal cells, mostly Kupffer cells, accumulated IT approximately 10 fold more, and were 100-fold more sensitive to IT, than parenchymal cells [119].

In a SCID mouse model of human xenografted $CD30^+$ ALCL (JB6 cell line), a 3-day treatment with non-toxic doses of Ber-H2/saporin (50% of LD_{50}) induced lasting complete remissions (CR) in 80% of mice, when started 24 hours after tumour transplantation. In contrast, injection of the IT at later stages of tumour growth (mice bearing subcutaneous tumours of 40- to 60-mm^3 volume), induced CR in only 6 of 21 (approximately 30%) mice and significantly delayed tumour growth rate [120]. A strong anti-tumour activity for Ber-H2/saporin was also observed in another SCID mouse model of ALCL (D430B cell line). A dose of IT of 0.1 mg/kg 48h after tumour transplantation gave a CR in 4/6 mice and a PR in 2/6 [121]. Main toxicity signs in mice and rabbits were dose-related increase of serum transaminases (AST and ALT) and creatine phosphokinase (CPK). LD_{50} (as RIP) in Swiss mice was 7 mg/kg for Ber-H2/MOM and 0.45 mg/kg for Ber-H2/PAP-S [118].

Six mAbs (termed Ki-2 to Ki-7) linked to RTA inhibited L540Cy protein synthesis with IC_{50}s ranging 10^{-10}-10^{-11} M. The most effective IT, Ki-4-RTA, was able to improve the mean survival time (MST) of lymphoma-bearing SCID mice from 42 days in untreated controls to more than 132 days [122].

1.11. Immunotoxins Targeting CD33

CD33 is an antigen found on myeloid leukaemia blasts and myeloid progenitor cells, but it is not expressed in detectable amounts on the ultimate haematopoietic progenitor stem cells.

An α-CD33 IT anti-MY9-bR was toxic to HL60 and to primary AML cells, but resulted toxic also to normal CFU-GM, which expresses CD33, and to BFU-e and CFU-C, although to a lesser extent [123]. Nevertheless, the concentration of long-term culture initiating cells (LTC-IC) in $CD34^+$ purified cells, quantified by a limiting dilution technique, was found to be increased following α-CD33-bR treatment [124].

Two α-CD33 ITs reactive with AML were constructed by conjugating gelonin to murine and humanised M195 mAb. Treatment of HL60 cells with HuM195-gelonin resulted in more than 1000 times lower colony formation; normal bone marrow mononuclear cell colony-forming units treated with IT at same dose were reduced by a factor of 10 [125]. Humanised M195 antibody linked to recombinant gelonin had significant tumour suppressive activity *in vivo* in a nude mouse model of human myeloid leukaemia. 50% of mice treated with HuM195-rGel did not develop leukaemic tumours for 5 months and the other 50% had significantly retarded tumour growth [126]. HuM195-rGel was toxic to AML cell lines and primary AML blasts obtained from patients and exposed to the immunotoxin *in vitro* [127].

1.12. Immunotoxins Targeting CD38

CD38 is strongly expressed by myeloma, lymphoma and B- and T-leukaemia cells obtained from patients. A limitation to the potential *in vivo* use of an α-CD38 immunotoxin is a rather wide expression of CD38 antigen by several cell types. A conjugate mAb HB7-blocked ricin had a low level of toxicity on haematopoietic progenitor cells. Tumour cells from four of five patients were 100-fold to 500-fold more sensitive to the inhibitory effect of HB7-blocked ricin than normal BM cells [128]. In a model of Ramos cells growing aggressively in SCID mice an α-CD38 IT (OKT10-saporin) led to a significant prolongation of survival as compared with controls; but all treated animals eventually succumbed to disease [129].

1.13. Immunotoxins Targeting CD40

CD40 is highly expressed in B-cell malignancies, including B-lineage leukaemias, NHL, Hodgkin's disease, and multiple myeloma.

A single-chain immunotoxin consisting of bryodin fused to the single-chain Fv region of the α-CD40 antibody G28-5 was potently cytotoxic against CD40-expressing B lineage from non-Hodgkin's lymphoma and multiple myeloma cell lines, with IC_{50} values in the ng/ml range. This IT did not result toxic to CD40-expressing carcinoma cell lines, which however, were sensitive to a bryodin-based immunotoxin conjugate targeted to the Ley carbohydrate antigen [130]. Antitumour activity was also observed in preliminary studies in which bryodin-G28-5 scFv was given to SCID mice bearing human non-Hodgkin's lymphoma and multiple myeloma xenografts [131].

1.14. Immunotoxins Targeting CD64

CD64, the high-affinity receptor for immunoglobulin G (FcγRI), is constitutively expressed on monocytes/macrophages and some dendritic cells, and it is induced on neutrophils by IFNγ or G-CSF. CD64 has been implicated in a number of diseases, such as infectious and auto-immune diseases, and cancer.

The α-CD64-RTA IT was proposed for the elimination of activated macrophages with the aim of resolving chronic inflammation. This IT was able to eliminate activated macrophages *in vitro* and *in vivo*, in a cutaneous inflammation model, induced in mice transgenic for human CD64 [132]. Inflammatory macrophages from synovial fluid obtained from rheumatoid arthritis patients expressed elevated levels of CD64 and were selectively eliminated by α-CD64-RTA IT via apoptotic cell death. Monocyte/macrophages from peripheral blood of the same patients, which had lower levels of CD64 expression, were much less affected [133].

Blast cells from patients with AML commonly express CD64. Cells from 60% of AML patients were inhibited by the combination of α-CD64-dgA IT with IFNγ, and this inhibition was found to correlate with CD64 expression. In a NOD/SCID mice model of human AML, α-CD64-dgA/IFNγ inhibited 95-98% of peritoneal exudate AML cell

proliferation and 85-90% of solid leukaemia masses. The effect of this IT on AML cells was dependent on activation of cells by IFNγ [134].

1.15. Immunotoxins Targeting Costimulatory Antigens

The most important regulatory signal for T-cells activation is the interaction between CD80 (B7-1) and CD86 (B7-2) on APC with CD28 and cytotoxic T-lymphocyte antigen-4 (CTLA-4) on T-cells. Much attention has been devoted to the manipulation of this costimulatory pathway for therapeutic purposes. Immunotherapy with monoclonal antibodies anti-costimulatory molecules could be utilised either for the suppression of the immune system (autoimmune diseases, transplant rejections and graft versus host disease-GVHD) or for its stimulation (vaccinations and tumours).

Immunotoxins Targeting CD80/CD86

A strong expression of the CD80 activation antigen has been described on most Hodgkin and Reed-Sternberg (H-RS) cells, independently of the histological subtype, whilst the CD86 antigen appears widely expressed on H-RS cells, particularly in the histological subtypes showing mixed cellularity or nodular sclerosis. An α-CD80 immunotoxin, B7-24-saporin, exhibited strong cytotoxicity against the $CD80^+$ B-cell line Raji, and the RS cell lines HDLM2 and KM/H2 (IC_{50}s $<10^{-11}$ M). In clonogenic assays with Raji cells or KM/H2 cells, a 3- or 4-log kill, respectively, was observed. No cytotoxicity was found against the B7-1 negative epithelial and endothelial cell lines or against haematopoietic progenitor cells [135].

Immunotoxins specific for the CD80 and CD86 antigens were prepared by linking three type 1 RIPs, namely bouganin, gelonin and saporin, to the monoclonal antibodies M24 (α-CD80) and 1G10 (α-CD86). These immunotoxins showed a specific cytotoxicity for the CD80/CD86-expressing cell lines Raji and L428. The immunotoxins inhibited protein synthesis by target cells with IC_{50}s ranging from 0.25 to 192 pM as RIPs. The α-CD80 ITs appeared 1-2 logs more toxic for target cells than the α-CD86 ones. Immunotoxins containing saporin and bouganin induced apoptosis of target cells. Bouganin and related ITs at concentrations up to 100 mM did not significantly affect the recovery of committed progenitors or of more primitive cells. The saporin-containing ITs at concentrations ≥ 1 nM showed some toxicity on CFU-C. However, the LTC-IC assay did not reveal any toxicity of saporin immunotoxins also at the highest concentration tested (100 nM) [136].

The effects of ITs composed of gelonin conjugated to mAbs against CD80 (5B5) or CD86 (1G10) were examined *in vitro* on CD80 and CD86 transfected cell lines. In primary mixed lymphocyte cultures (MLCs), the average inhibitory capacity of α-CD86-IT (72%) and α-CD80-IT (30%) was significantly higher than that of mAbs alone. These results therefore demonstrate that αB7-ITs functionally block B7-CD28 costimulatory signalling and eliminate activated APC [137]. The pharmacokinetics of this α-CD86-IT were evaluated in rhesus monkeys. α-CD86-IT was administered as single intravenous bolus injection, and achieved a plasma concentration 50-fold exceeding that required to eliminate cultured Hodgkin/Reed-Sternberg cells within 6 h. The animals were capable of generating primary immune responses to both gelonin and murine IgG within 9 days after infusion with α-CD86-IT [138].

Immunotoxins Targeting CD152 (CTLA-4)

CTLA-4 (CD152) co-stimulatory molecule, a homologue of CD28, plays an inhibitory role in regulating T cell response by interacting with the common ligands CD80/CD86 on APC. The CTLA-4 expression is induced on T cells upon activation, followed by a rapid internalization, and its membrane expression is restricted to activated T cells. Thus, this antigen represents a good target for T cell immunotherapy, in particular of organ rejection and GVHD following hemopoietic stem cell transplants.

Immunotoxins containing recombinant human-derived α-CTLA-4 scFv (83 and 40) linked to saporin induced apoptosis in activated T lymphocytes and were able to specifically inhibit MLRs between T lymphocytes and dendritic cells, and between T lymphocytes and an EBV-positive lymphoblastoid B cell line. The most effective immunotoxin (83-saporin) tested on CD3/CD28-stimulated lymphocytes had an IC_{50} of 7.83×10^{-11}M and an AC_{50} (concentration causing apoptosis in 50% of cells) of 2.33×10^{-12}M [139,140]. No toxicity for haemopoietic precursors was reported. 83-saporin was tested in a model of tumour rejection consisting of C57BL/6 mice bearing a murine H.end endothelioma cell line, derived from DBA/2 mice. The lymphoid infiltration due to the presence of the tumour was markedly reduced, demonstrating that the immunotoxin was actually available and active *in vivo* [140]. Recently, the surface expression of CTLA-4 in a variety of acute and chronic myeloid leukaemias (AMLs and CMLs) and B- and T-lymphoid leukaemias, either adult or paediatric, was demonstrated. The anti-CTLA-4 immunotoxins were able to induce *in vitro* apoptosis of neoplastic cells from AML, with AC_{50}s in 10^{-11} M range [141].

1.16. Cocktails of Various Anti-lymphocytes Immunotoxins

Immunotoxins composed by 35.1 (α-CD2), T101 (α-CD5), G3.7 (α-CD7) or TA-1 (α-gp95,170) mAbs and whole ricin were used alone or in combination. These immunotoxins, at concentrations greater than 300 ng/ml and in the presence of lactose, were able to induce a rapid protein synthesis inactivation of leukaemic cell lines, with subsequent inhibition of clonogeneic growth (4 logs of inhibition for an equimolar immunotoxins cocktail), sparing the majority of committed (CFU-GM, CFU-E) and pluripotent (CFU-GEMM) haematopoietic stem cells [17]. The same ricin conjugates containing G3.7, T101 and 35.1 mAbs were used to elucidate the immunobiologic features of leukaemic progenitor cells in T-lineage acute lymphoblastic leukaemia (T-ALL) [142].

van Oosterhout *et al.* [143] reported a preclinical study with an α-CD3/α-CD7 cocktail of RTA-containing immunotoxins potentially useful for the treatment of GVHD. The LD_{50} of the immunotoxins combination was 25-45 mg IT/kg mouse. In cynomolgus monkeys clinically significant serum concentrations were obtained without irreversible toxicities occurring. The $t_{1/2}$ varied between approximately 6 and 9 h and the C_{max} ranged from 1.8 to

3.9 μg/ml. The main side effect was a transient rise of serum creatine kinase.

The α-CD7 and α-CD38 $F(ab'\gamma)_2$ BsAbs were used, alone or in combination, for delivering saporin on the cell surface of human T-ALL cell line. Used alone against HSB-2 cells, the α-CD7 BsAb HB2 × DB7-18 and the α-CD38 BsAb OKT10 × RabSap gave a 435- and a 286-fold increase of saporin toxicity, respectively. The combination of the two BsAbs on the same cells was 10-fold more effective than the best single BsAb, with an increased rate of protein synthesis inactivation [48]. Treatment of SCID/CEM mice with a single IT containing the parental murine IgG1 α-CD7 or α-CD38, led to a delay in the development of leukaemia, but 90% of animals treated with either IT developed disseminated leukaemia cell growth. The combination of HB2-SAP (α-CD7) and OKT10-SAP (α-CD38) ITs, administered in 3×10 μg i.v. doses, significantly increased the surviving time and the number of leukaemia-free animals (60%) [144].

α-CD19 HD37 mAb and α-CD22 RFB4 mAb, conjugated with dgA, killed 4 logs and 2 logs of Daudi tumour cells transplanted into SCID mice, respectively. The anti-tumour activity of the two ITs administered in combination was significantly enhanced in SCID/Daudi mice and was consistent with the killing of greater than 5 logs of tumour cells, but still the treatment was not curative [76]. The disease was completely eradicated when the two ITs cocktail were combined to any of three chemotherapeutic drugs (doxorubicin, cytoxan, or camptothecin) and given the day following tumour cell inoculation [145]. The same combination therapy in mice with advanced tumour significantly increased their survival, but was not curative. The best anti-tumour effect was achieved when the ITs cocktail was given before or at the same time as chemotherapy, whilst no additional therapeutic benefit was obtained by giving IT after chemotherapy [146]. When tested *in vitro* on primary culture of patient-derived pre-B ALL cells, HD37-dgA and RFB4-dgA ITs cocktail, further named Combotox, also induced a statistically significant reduction in the number of viable cells, resulting more effective than each single IT [77].

SCID mice challenged with the $CD19^+$ $CD38^+$ human Burkitt's lymphoma cell line Ramos treated with three daily injections of α-CD19 BU12-saporin IT or of α-CD38 OKT10-saporin IT, starting at day +7, had a significantly prolonged survival. When both ITs were used in combination at equivalent dose levels, the therapeutic outcome was significantly improved over that obtained with single IT therapy, with 20% of animals surviving disease-free at 300 days [88]. Similar or even better results were obtained, in the same Ramos/SCID mice model, with a combination of three α-CD19, α-CD22, and α-CD38-saporin immunotoxins [147].

1.17. Immunotoxins with Bispecific Antibodies with Double Cell Antigen Specificity

Three bispecific α-CD22/α-CD3 ITs were generated and tested against Daudi target cells. Parental IT, Fab'-α-CD22-dgA made a potent immunotoxin for B cells, but not T cells, while Fab'-anti-CD3-dgA did not kill either T or B cells. All the three BsAbs induced LAK-T cell-mediated specific lysis of $CD22^+$ Daudi cells. Two of the purified BsAbs were conjugated to dgA to produce BsITs. In the presence of LAK-T cells, the BsITs were 3- to 17-fold more cytotoxic than unconjugated BsAbs to Daudi cells, and showed the highest antitumour activity with IC_{50}s 2.1-3.2 $\times 10^{-11}$ M [148].

An immunotoxin, made by α-CD4/α-CD26 bispecific antibody and blocked ricin, was found to be specifically toxic to $CD4^+CD26^+$ memory T-cells, which provide help for B-cell Ig synthesis. This IT was effective on lymphocytes activated with PHA or through a MLR, but had a minimal effect on resting T cells [149]. The same authors developed an α-CD4/α-CD29-bR IT that was able to kill $CD4^+$CD29bright T cells. Both CD4/CD26 and CD4/CD29-directed ITs were proposed for a selective removal of activated T cells from, or directed against, tissue grafts.

1.18. Immunotoxins Toward Multy-drug Resistant Cells

As previously described an α-CD19 IT (anti-B4-bR) combined to doxorubicin or etoposide produced supra-additive effects, *in vitro*, on the P-glycoprotein-expressing cell line Namalwa/mdr-1 and significantly increased the life span of Namalwa/mdr-1 challenged SCID mice. After treatment with anti-B4-bR, the multi-drug resistant Namalwa/mdr-1 population expressed lower levels of P-glycoprotein, rendering these cells more sensitive to doxorubicin [85]. Different results were obtained with the α-CD138 B-B4-saporin IT**,** which did not influence the sensitivity of drug-resistant RPMI8226 to doxorubicin. Moreover, this IT resulted equally toxic to drug-sensitive and drug-resistant cells from patients with multiple myeloma, and less toxic to drug-resistant with respect to drug-sensitive RPMI8226 cells [108].

The RV+ cell line, which is a P-gp-expressing variant of the human myeloid leukaemic cell line HL60, displayed a typical MDR phenotype and a relative resistance to HuM195-gelonin α-CD33 IT and to free rGelonin. This resistance was demonstrated to be related to P-gp expression, as K562 leukaemia cells retrovirally infected to overexpress P-gp also were found to be resistant. Confocal microscopy showed that HuM195-gelonin was accumulated preferentially in the lysosome in RV+ cells but not in sensitive HL60 cells, suggesting that increased lysosomal degradation was most likely responsible for the resistance to this IT in MDR cells [150].

In spite of these conflicting results a selective elimination of multidrug resistance-positive cells (LoVo/Dx) was obtained by using the monoclonal antibody MRK 16, which recognises a surface epitope of the 170 kDa P-glycoprotein, and a sheep α-mouse IgG antibody, conjugated to saporin. With this system, after only 2 h of incubation with the α-mouse-IgG IT, over 2-logs of cell killing was obtained and no residual MDR^+ colonies were still detectable in a LoVo/Dx enriched bone marrow [151].

2. IMMUNOTOXINS TARGETING GROWTH FACTOR RECEPTORS

It is well known that growth factors play an important role in normal cell proliferation by means of stimulation of

growth factor receptors located on the surface of cells. Tumour cells express high levels of growth factor receptors that theoretically can serve as therapeutic targets in cancer treatment.

2.1. Immunotoxins Targeting EGFR

The overexpression and aberrant function of the epidermal growth factor receptor (EGFR, HER1) and its ligands and coreceptors in a wide spectrum of cancers have provided a rationale for targeting this signalling network with novel treatment approaches, such as immunoconjugates.

Initially conjugates were made with the growth factor molecule, with encouraging results [152,153]. Many different immunotoxins were subsequently obtained by linking RIPs to α-EGFR monoclonal antibodies. An immunoconjugate prepared with mAb 528 and recombinant ricin A showed dose-dependent killing of cells with 10^5 EGFR/cell or more [154]. The B4G7-gelonin conjugate was specifically cytotoxic for cells overexpressing EGFR and, in nude mice, was capable of suppressing the growth of an EGFR-hyperproducing carcinoma cell (A431) solid tumour [155].

Two immunoconjugates, Mint5-ocymoidine and Mint5-pyramidatine, exerted specific cytotoxicity on EGFR expressing target cells with IC_{50}s in a 10^{-11}-10^{-12} M range. Both ITs inhibited the growth of grafted human tumour cells (A431) in nude mice [156]. An indirect α-mouse IgG-saporin IT caused a significant inhibition in cell growth and protein synthesis and a strong increase in apoptosis in rhabdomyosarcoma cells (RD/18 cell line) pretreated with the α- EGFR 528 mAb [157].

Heparin-binding epidermal growth factor (HBEGF) binds to EGFRs with high affinity and to heparan sulfate proteoglycans, resulting in increased mitogenic potential compared to other EGF family members. Recombinant fusion proteins consisting of mature human HBEGF fused to saporin was cytotoxic *in vitro* to a variety of EGFR-bearing cell lines and inhibited growth of EGFR-over-expressing human breast carcinoma cells *in vivo* [158].

Another carrier to deliver toxin to EGFR is the transforming growth factor-alpha (TGFα), which conjugated to RTA resulted highly cytotoxic on A431 cells [159].

Her-2/neu (HER-2) is overexpressed by a variety of tumours such as breast, ovarian, gastric, and colorectal carcinomas. Overexpression of this oncogene is directly associated with malignant transformation of epithelial cells. The frequency of HER-2 overexpression varies among the different types of cancers, but universally represents a marker of poor prognosis. The critical role of HER-2 in epithelial oncogenesis as well as its selective overexpression on malignant tissues makes it an ideal target for immunotherapy. Several researchers tested its potentialities as target for cancer therapy. Tumour sensitivity to an α-HER-2-ricinA immunotoxin seems to depend on cell surface antigen density [160]. In tissue distribution studies, the BACH-250-recombinant gelonin IT showed a 2-10-fold higher concentration in tumours than in normal tissues, with optimal tumour uptake occurring 48-96 h after administration. Plasma clearance curves for BACH-250/rGEL showed terminal-phase half-lives of 72 h. Moreover, the IT resulted active *in vivo* in human tumour xenograft models [161], whereas indirect immunotoxins against HER-2 and HER-3 were ineffective on human rhabdomyosarcoma cells [157].

2.2. Immunotoxins Targeting FGFR

Fibroblast growth factor receptors are a family of at least 12 different proteins. Many solid tumours express basic FGF (or FGF2, or bFGF) receptor. The conjugate FGF2-saporin was toxic, *in vitro*, for human melanoma, teratocarcinoma, and neuroblastoma cells expressing FGF-receptors. Mice treated with FGF2-SAP showed dramatic tumour growth inhibition with minimal toxicity [162,163]. A FGF-saporin conjugate demonstrated potent cytotoxicity in malignant bladder cell lines with an IC_{50} range 0.13-13.6 nM, whereas cells derived from normal fetal bladder were less sensitive to FGF2-saporin (IC_{50} >100 nM) [164]. The number of high-affinity FGF receptors, and FGF2 cellular content and secretion are not absolute determinants of cellular sensitivity to FGF2-saporin. Recombinant FGF2-SAP, though less cytotoxic than its chemical conjugate form, effectively targeted tumour cells xenografts in nude mice, in an animal model of prostate cancer, and induced dramatic reduction of large, established tumours [165].

Basic fibroblast growth factor is also an important mediator of smooth muscle cell (SMC) proliferation following arterial injury that results in neointimal growth. The effect of the conjugate rFGF2-SAP was evaluated on venous anastomotic intimal hyperplasia, a major cause of failure of arteriovenous (AV) graft. When polytetrafluoroethylene-based local infusion devices were implanted bilaterally as femoral AV conduits in dogs, local infusion of rFGF2-SAP significantly reduced venous anastomotic intimal hyperplasia and cell proliferation, without causing systemic toxicity [166,167]. In a canine model of unique co-culture, which better replicates the relationship between SMC and vascular endothelial cells (EC) *in vivo*, was demonstrated a dose-response range of rFGF2-SAP at which both the proliferation and total cell number of SMC, but not EC, was significantly reduced [168].

3. CONJUGATES TARGETING TRANSFERRIN RECEPTOR

Transferrin receptor (TfR) is expressed on all normal tissues, but it is chosen as target of immunotherapy for glioma and other tumour cells because of the high iron requirement of rapid proliferant neoplasms. Immunotoxins targeting the TfR were hypothesized to possess sufficient specificity to eliminate neoplastic cells in the central nervous system or other compartments where delivery of immunotoxins to tumour would not require transvascular transport. Two different conceptual approaches have been designed for targeting TfR on cancer cells. Immunotoxins were made using as carrier the plasma protein transferrin and, alternatively, α-TfR monoclonal antibodies. Both type of proteins can be used to deliver RIP on target cells, however, Tf-based conjugates have two major disadvantages on mAb based conjugates as they are strongly influenced by the

presence of free transferrin and by the iron saturation state [169].

3.1. Transferrin-RIP Conjugates

A transferrin-saporin conjugate was found to be internalized via binding to TfR. The conjugate displayed an inhibitory activity on K562 (human erythroleukaemia) cell proliferation and, in a clonogenic assay, a marked inhibition of K562 colony formation (about 100% at 10^{-9} M) [170]. In healthy rat the immunotoxin Tf-RTA did not accumulate following i.t. inoculation, reaching non-toxic concentrations in the brain tissue and in the peripheral blood, whereas in the cerebrospinal fluid as well as at the interface cerebrospinal fluid/brain tissue the immunotoxin may reach potentially therapeutic concentrations. The clearance of Tf-RTA from the cerebrospinal fluid was rapid (9.1 µl/min), the IT then diffused into the brain tissue and in the peripheral blood. The rate of immunotoxin elimination from the peripheral blood following either i.v. or i.t. administration was similar (kel = 0.0021, 0.0025 min^{-1}, respectively) [171].

The non-toxic type-2 RIPs Nigrin b and ebulin l were used to construct transferrin-conjugates. Conjugation with Tf decreased the IC_{50} of the proteins from 3×10^{-7} M (nigrin b) and 1.5×10^{-8} M (ebulin l) to 3.5×10^{-10} M in HeLa cells [172]. In another original approach, transferrin was conjugated to a chimeric toxin (cRTA, a fusion of RTA gene and a DNA fragment of protein G of the vesicular stomatitis virus). This conjugate showed 10-20-fold greater cell killing efficacy than Tf-nRTA or Tf-rRTA conjugates, despite equivalent binding of the three conjugates to target tumour cells [173].

Conjugated RIP-transferrin were also used in other therapeutic approach, such as in malaria. Gelonin linked to human transferrin was targeted to *Plasmodium falciparum*. The transferrin toxin conjugate was significantly toxic to parasite growth and resulted 25 times more potent than the toxin alone in inhibiting parasite protein synthesis [174].

3.2. Immunotoxins Targeting TfR

Immunotoxins constructed by linking RTA to mAbs reactive with TfR have been evaluated in a nude mouse model of human malignant mesothelioma. The survival of tumour-bearing mice was extended by 149-404% [175].

The α-TfR conjugate 5E9-gelonin, when administered i.v. at the time of i.p. tumour inoculation, prolonged survival of nude mice bearing the Burkitt's lymphoma Namalwa cells or other human tumours as ascites xenografts and delayed or prevented the growth of subcutaneous nodules of Namalwa cells in an antigen-specific fashion after a single i.v. injection. Direct intratumoural administration also inhibited the growth of visible subcutaneous nodules of Namalwa cells. Pharmacokinetic analysis of the disappearance of this immunoconjugate from the murine circulation revealed that it had a biphasic clearance, with an initial rapid phase with $t_{1/2}$ of 3 h and a later, slower phase with a $t_{1/2}$ of about 1 day. Analysis by SDS-PAGE of blood samples revealed that a substantial disulphide-linker breakdown occurred *in vivo* [176].

An α-TfR-ricin immunotoxin was tested *in vitro* and resulted highly potent and cell type-specific in killing cells derived from human glioblastoma, medulloblastoma, and leukaemia [177].

The efficacy of the combination of doxorubicin with an α-TfR-RTA immunotoxin was determined *in vitro*, on H-MESO-1 human malignant mesothelioma cells, and *in vivo*, on the same cells i.p. injected in nude mice. The effect of doxorubicin on the antitumour activity of immunotoxin was directly compared to that of monensin, an immunotoxin potentiator. In animal trials, the immunotoxin combined with monensin or doxorubicin produced an increase in survival. The use of all three agents produced an additional improvement in survival [178].

The efficacy and cytotoxic properties of an immunotoxin α-TfR-rRTA were examined in cell lines and surgical specimens from paediatric brain tumours. Three target medulloblastoma cell lines (DAOY, D283MED, and D341MED), a glioblastoma (U373), and a neuroblastoma (SH-SY5Y) cell lines exhibited similar sensitivity to the immunotoxin with IC_{50}s in the 10^{-9}-10^{-10} M range. Experiments on fresh specimens of paediatric brain tumours treated with IT revealed that the more aggressive and malignant tumour types, such as glioblastoma multiform and medulloblastoma, were extremely sensitive to transferrin receptor-targeted immunotoxins, with IC_{50} values in the 10^{-12} M range. In general, protein synthesis in slow-growing and benign tumours was not so greatly affected by immunotoxins [179].

The antitumour activity of the 454A12-rRTA immunotoxin administered intracavitary to nude mice 5 days after tumour cell injection was enhanced by a non-therapeutic dose of recombinant human interferon alpha. The synergy between immunotoxins and IFN-alpha is dependent on tumour burden. These agents are less effective against large tumour burdens (i.e. advanced stage disease), but their beneficial effects re-emerge after cytoreduction by combination chemotherapy [180]. The pharmacokinetics of the 454A12-rRTA within the cerebrospinal fluid of monkeys showed a biphasic clearance with an early-phase $t_{1/2}$ of 1.4 h and a late phase $t_{1/2}$ of 10.9 h. The clearance was 4.4 ml/h, or approximately twice the estimated clearance due to bulk flow of cerebrospinal fluid. Loss by degradation was ruled out because immunoblot analysis showed that the immunotoxin was stable for up to 24 h after administration [181].

The cytoreductive effects of α-TfR conjugates (Tf-RTA and OKT9-RTA) against tumour micromasses have been also evaluated in a multicellular tumour spheroid (MTS) model of human breast (MCF7) and glioblastoma (U118) tumour cells and with rat glioblastoma (9L) cells. Within the range of IT concentration resulting in partial inhibition of MTS growth, the sensitivity of treated MTS was extremely heterogeneous (the cytoreductive effects varying between 0.1 and 4 logs of cells killed), indicating that the efficacy of IT against 3-D tumours is heavily influenced by the number of target Ag expressed by the tumour cells, as well as by the affinity of IT/toxin-cell interaction [182,183]. Immunotoxins are generally less effective against MTS than against monolayer cells at equivalent conditions, as it was also demonstrated with the α-TfR immunotoxin OKT9-

gelonin. An experimentally verified model was used to define the conditions which lead to large penetration barriers. In general, transport barriers in MTS become more important as immunotoxins become more effective against cells grown as monolayers [184].

The antitransferrin receptor immunotoxin 454A12-rRTA had a potent antiproliferative effects on proliferating human ocular cells *in vitro* [185], and appeared a promising tool for the treatment of ocular proliferative diseases, such as proliferative vitreoretinopathy [186], bleb scarring after trabeculectomy, or corneal epithelial downgrowth syndrome. After injection into the vitreous cavity of vitrectomized rabbit eyes and into the anterior chamber or subconjunctival space of eyes not vitrectomized, the immunotoxin was measured by ELISA in aqueous, vitreous, and plasma. 454A12-rRTA IT was cleared rapidly from the vitreous cavity over the first 24 h ($t_{1/2}$ = 8.0 h), and thereafter was slow. The IT recovered from the vitreous cavity retained significant antiproliferative activity at 96 h. The immunotoxin was cleared within 6 h from the aqueous after anterior chamber injection, and was not present inside the eye after subconjunctival injection. The plasma did not contain immunotoxin after any of these injections [187].

4. IMMUNOTOXINS TARGETING SOLID TUMOUR ANTIGENS

4.1. Immunotoxins Targeting Melanoma Antigens

The high-molecular-weight melanoma-associated antigen (HMW-MAA) is expressed on a large majority of melanomas but not on most normal or other neoplastic tissues. The mAb 9.2.27, which binds with high affinity and specificity to the HMW-MAA, was linked to whole abrin and ricin. Molecular species with exposed binding sites on their B-chains were removed by a Sepharose 4B chromatography. Several melanoma cell lines showed differential sensitivity to these ITs. The abrin conjugate was far more toxic to the target cells than the corresponding ricin conjugate [188]. A 9.2.27-gelonin IT exhibited similar potency, better selectivity, better tumour localisation, and more significant therapeutic effects. However, in nude mice bearing established palpable melanoma, a significant therapeutic effect of the conjugate was found with multiple but not single dose i.v. treatment [189].

Another mAb recognising HMW-MAA ZME-018 was conjugated to gelonin. The ZME-gelonin conjugate was 10^6-fold more active than gelonin in inhibiting the growth of melanoma cells in culture [190]. Pharmacokinetic studies of ZME-gelonin demonstrated a plasma half-life of 20.6 h. Treatment of nude mice bearing well-developed s.c. A375 melanoma xenografts with IT resulted in suppression of tumour growth. In a murine model for a rapidly growing lethal metastatic human melanoma, treatment with ZME-gelonin resulted in a mean survival of 213% over controls [191]. The IT comprising recombinant gelonin demonstrated identical *in vitro* cytotoxic effects as the IT containing the native RIP and showed an improved *in vivo* pharmacokinetic ($t_{1/2}$ 42 h) and tissue distribution [192].

XOMAZYME-MEL, an antimelanoma mAb-RTA immunotoxin, was well tolerated and produced only minimal signs of toxicity in rats treated with a low IT dose (1 mg/kg/day for 14 days). Side effects in animals receiving the high dose (5 mg/kg/day) consisted of transient weight loss, peripheral edema, leukocytosis, hypoalbuminemia, and mildly elevated liver function tests. Histological findings in these animals included cytoplasmic vacuolisation of hepatocytes, focal myocardial, skeletal muscle degeneration and renal deposits of proteinaceous casts [193].

The mAb Ep2, recognising an epitope of HMW-MAA, was conjugated to saporin. Ep2/SAP IT efficiently killed antigen expressing cells with an IC_{50} 10^{-10} M approximately [194].

4.2. Immunotoxins Targeting Carcinoembryonic Antigen

Several anti-carcinoembryonic antigen (CEA) immunotoxins constructed with the A chain of ricin resulted potent and specific for CEA-bearing cells, suggesting that the CEA molecule is capable of directing productive internalization of ricin A chain. Comparison of the IC_{50}s of each immunotoxin on three target cell lines showed that the immunotoxin made with the monoclonal C-19 antibody was generally 6-7 fold more cytotoxic than the goat and baboon polyclonal antibody immunotoxins. The affinity of CEA-binding antibody is probably an important, but not a sole factor in determining the immunotoxin potency [195]. The α-CEA mAb C110 resulted an interesting carrier to obtain potent therapeutic immunoconjugates for human colon cancer. In *in vitro* protein synthesis inhibition assays, ^{90}Y-labelled C110-RTA IT was approximately 3.7-fold more toxic to the LS174T human colon carcinoma cell line than unmodified C110-RTA IT and 1380-fold more toxic than ^{90}Y-labelled C110 mAb. Biodistribution studies of ^{90}Y-IT in LS174T tumour-bearing mice showed that, at 24 h following i.p. injection, high accumulation of radioactivity was seen in tumour and liver and, thereafter, accumulation remained almost unchanged until up to 168 h (15-18% of injected dose/g in tumour and 10-15% in liver). The radioactivity in the spleen and bone reached highest levels (approximately 8%) at 168 h. Estimation of absorbed radiation doses to the tissues showed that i.p. tumour would have received approximately 1.5 to 7-fold higher radiation dose than normal organs [196].

4.3. Immunotoxins Targeting Other Antigens on Solid Cancer

The mAb SEN31, which recognises the cluster-5a antigen on small-cell lung cancer (SCLC) cells, was used to prepare a blocked ricin immunotoxin. In a series of experiments *in vitro* and in a SCLC xenograft model in nude mice, the immunotoxin SEN31-bR was potently and selectively active against SCLC cell lines [197]. SEN7 recognises a novel epitope on the neural cell adhesion molecule (NCAM), which is highly associated with SCLC. The immunotoxin SEN7-bR was selectively and potently active against a number of SCLC, with IC_{50} values ranging between 7 pM and 62 pM. Intoxication by immunotoxin proceeded rapidly following by a 2 h lag phase; the initial rate of protein synthesis inhibition occurred with $t_{1/2}$ value of 5.5 h [198].

The SWAII-dgA immunotoxin, recognising the leukocyte-differentiation antigen CD24, was evaluated

against SCLC cell lines and in 2 nude-mouse models. SCLC cells were selectively eliminated in clonogenic assays. In nude mice, SWAII-dgA was cleared from blood circulation with biphasic kinetics: an initial alpha phase of 1 h and a second beta phase of 20.5 h. The i.v. injection of a dose of IT equivalent to 30% of the LD_{50} delayed the growth of SW2 solid-tumour xenografts by 16 days [199].

An N901-blocked ricin (N901-bR) immunotoxin against CD56, an antigen of the family of NCAMs, exhibited specificity for several neoplasms of neuroectodermal origin, including SCLC and neuroblastoma. Reactivity with normal tissues was essentially limited to various neuroendocrine cells, cardiac muscle cells, and cells in peripheral nerve tissue. Tumour cells *in vitro* were specifically eliminated by exposure to N901-bR. The immunotoxin under conditions optimal for *in vitro* tumour cell depletion was not toxic to haematopoietic precursors [200].

ZENECA ZD0490 is a recombinant RTA-containing immunotoxin that recognises the antigen CA242, expressed on approximately 65% of colorectal tumours. ZD0490 was extremely potent against colorectal tumour cell lines CoLo201 and CoLo205, which express the CA242 antigen. ZD0490 activity was determined *in vitro*, by both protein synthesis inhibition and clonogenic assay, as well as *in vivo*, where single dose i.v. administration of 2.5 mg/kg was sufficient to induce substantial growth delay of both CoLo201 and CoLo205 s.c. tumours in nude mice [201]. The pharmacokinetics of this immunotoxin was studied in mice. Following a single i.v. bolus dose of 2.5 mg/kg (50% LD10), the clearance from plasma was extremely slow: 34 μL/min/kg, $t_{1/2}$ β=33 h. Repeated doses of IT (1 mg/kg day ×5) did not lead to drug accumulation [202].

The monoclonal antibody ONS-M21 recognising a human astrocytoma- and medulloblastoma-associated antigen, was conjugated to RTA. A cytotoxic effect against ONS-76 cells was found, but not against antigen-negative HuH-7 and SW480 cells [203]. A humanised ONS-M21 antibody (hM21) was engineered as scFv (schM21), and its ability to internalise into tumour cells was evaluated by conjugation with RTA. The conjugate inhibited the growth of ONS-76 target cells [204].

Four mAbs, including 14G2a (α-disialoganglioside), ch14.18 (a humanised switch variant), BW704 (α-disialoganglioside), and chCE7 (α-glycoprotein of M*r* 190,000), were subsequently linked to deglycosylated RTA. The most potent immunotoxin, 14G2a-dgA, inhibited the protein synthesis of neuroblastoma cell lines IMR5 and NMB by 50% at 6×10^{-12} M concentration. The antitumour efficacy of these immunotoxins was tested in a disseminated human neuroblastoma model in SCID mice. Treatment of tumour-bearing mice with 14G2a-dgA 12 days after tumour challenge resulted in a significant prolongation of survival as compared with controls (16.8 versus 6.5 weeks) [205].

The antigen GD2 is selectively expressed on the surface of neuroblastoma cells, and is detected by the monoclonal antibody BW704, which was conjugated to deglycosylated RTA. The mean survival time of BW704-dgA treated animals was significantly increased (49 days) compared to the control animals treated with an irrelevant immunotoxin, unconjugated BW704, or control buffer (33 to 39 days) [206].

Three monoclonal antibodies (J591, PEQ226.5, and PM2P079.1) against prostate-specific membrane antigen (α-PSMA) were linked to RTA and their cytotoxic effects were investigated in monolayer and three-dimensional (3-D) cell cultures of prostate carcinoma cells (LNCaP). The immunotoxins showed effects at nM concentration. Treatment with J591-SMPT-nRTA (0.35-31.7 ng/ml) resulted in complete eradication of 3-D tumour micromasses or in 1.46- to 0.35-log reduction of target cells number, depending on the dose [207].

The 48-127 mAb recognises a glycoprotein (gp54) expressed on all human bladder tumours and on normal urothelium, but not on the luminal surface of normal urothelial umbrella cells. Immunotoxins prepared by linking the type 1 RIP momordin I, PAP-S and saporin to 48-127 were specifically toxic to T24 cells (bladder cancer derived) with IC_{50}s at nM concentrations. The inhibition of protein synthesis in the T24 cells required a 2 h contact with immunotoxins to ensure optimal binding and endocytosis [208].

4.4. Immunotoxins Targeting Tumour Vasculature

Vascularisation is an important step in tumour growth and metastasis. Tumour neovascularisation can be considered, therefore, a good target for antineoplastic therapy. Targeting ITs to endothelial cells would overcome the problem of difficult penetration of ITs into solid tumour masses. An α-tumour endothelial cell immunotoxin could cause complete occlusion of the tumour vasculature and dramatic necrotic events in large solid tumours.

P1GF is an angiogenic factor involved in tumour neovascularisation. The fusion protein P1GF-2-saporin showed selectivity and anti-tumour activity [209].

Endoglin (CD105) is a proliferation associated antigen on endothelial cells. SN6f, SN6j and SN6k mAbs, specific for the human CD105, and cross-reacting with murine endoglin, were conjugated to dgA. All ITs showed a weak but specific cytotoxic activity on target cells with IC_{50}s 29, 16 and 26 nM, respectively. These immunotoxins showed a significant anti-tumour efficacy and antiangiogenic activity in SCID mice inoculated with human tumours [210,211]. Vascular endothelial growth factor (VEGF) plays a key role in the growth and metastasis of solid tumours. A fusion protein containing VEGF linked to gelonin had anti-tumour activity against human melanoma (A-375) or human prostate (PC-3) xenografts, with a reduction in tumour volume to 16% of untreated controls [212].

5. IMMUNOTOXINS TO HIV-INFECTED CELLS

The objective of using ITs to treat AIDS is the elimination of HIV-infected lymphocytes that by secretion of viruses actively spread the infection. Two strategies can be used to this aim: immunotoxins targeting viral antigens or immunotoxins targeting lymphocytes antigens.

5.1. Immunotoxins Targeting Lymphocyte Antigens

Two different populations of infected T cells are present in HIV-infected individuals: activated cells that produce

virions and quiescent cells that harbour the viral genome but are unable to produce virus unless they are activated. The alpha-chain of the IL-2R (CD25, Tac, p55) is expressed on activated but not resting T cells and therefore represents a good marker to distinguish activated from resting T cells. An α-CD25-RTA immunotoxin eliminated activated, $CD25^+$ HIV-infected cells and, thereby, inhibited viral production by these cells. Subsequent addition of cyclosporine to the residual $CD25^-$ cells prevented their activation and thereby suppressed their ability to produce virus and to propagate the infection to uninfected T cells [213]. An α-CD45RO-dgA IT was proposed to eliminate the $CD25^-$ latently infected cells [214]. *Ex vivo* treatment of cells from HIV-positive persons by α-CD45RO-dgA IT reduced the frequency of both productively and latently infected cells [215].

The toxicity and pharmacokinetics of an α-CD7 immunotoxin (TXU-PAP) were evaluated in HIV-infected chimpanzees. At a total dose of 100 μg/kg, TXU-PAP did not cause severe (grade ≥3) toxicity in any of the four HIV type 1 (HIV-1)-infected or two healthy chimpanzees. The only side effects were a transient elevation of the liver enzyme alanine aminotransferase between days 2 and 14 without a concomitant rise in total bilirubin levels and a decrease in the serum albumin levels between days 1 and 5 without any concomitant weight gain or peripheral edema. TXU-PAP showed favourable pharmacokinetics in chimpanzees with a plasma elimination half-life of 5.1 to 12.0 h and a systemic clearance of 5.8 to 15.1 ml/h/kg. At 2 months after initiation of the TXU-PAP infusions, the HIV-1 burden was reduced to below-detection levels in three of the four chimpanzees, and in the remaining chimpanzee, the HIV burden was <500 RNA copies/ml at 2 weeks, but returned to the pretreatment levels by 2 months [216].

5.2. Immunotoxins Targeting Viral Antigens

The gp120 envelope glycoprotein of the human immunodeficiency virus (HIV), which is expressed on the surface of many HIV-infected cells, binds to the cell surface molecule CD4. Soluble recombinant CD4 conjugated to RTA killed HIV-infected H9 cells, was 1000 fold less toxic to uninfected H9 cells and was not toxic to Daudi cells (which express major histocompatibility class II antigens, the putative natural ligand for cell surface CD4) [217]. Two murine antibodies, recognising the CD4-binding region, and a variable region of gp120, were chemically coupled to PAP-S. Both immunoconjugates specifically killed human T cells H9, infected with HIV-1. The immunoconjugates retained the ability to neutralise HIV virions to infect T cells and to prevent the syncytium formation [218]. Polyclonal human α-gp160 antibodies were used to produce an immunotoxin with the broadest specificity for different HIV strains and the greatest specific activity. This is related to the polyclonal nature of the preparation rather than to an increase in relative avidity of the antibody [219].

Immunotoxins that bind epitopes exposed on the cell surface effectively killed persistently infected cells, although killing was not directly proportional to the amount of immunotoxin bound to the cell. The activity of α-gp41, but not α-gp120, immunotoxins was markedly enhanced in the presence of soluble CD4 or peptides corresponding to the CDR3 region of CD4. CD4-mediated enhancement of α-gp41 immunotoxin activity was observed for laboratory strains neutralised by sCD4 and for clinical isolates that were resistant to neutralisation by sCD4 [220, 221].

6. IMMUNOTOXINS TARGETING ACETYLCHOLINE RECEPTOR

Immunotoxins directed against acetylcholine receptor (AChR) were initially made for experimental therapy of myasthenia gravis (MG) and other autoimmune diseases. The pathogenesis of MG involves a T cell-dependent antibody-mediated autoimmune response directed against AChR. Inactivation of AChR-specific T cells should interrupt the immune response, resulting in therapeutic benefit. Antibody responses by rat lymph node cells against purified acetylcholine receptor (AChR) were found to be inhibited by protein conjugates prepared with anti-idiotypic antibody and RTA [222,223]. Rats suffering from experimental autoimmune myasthenia gravis (EAMG) induced by previous immunisation with foreign AChR were treated with AChR-gelonin conjugates. This led to a marked improvement of clinical symptoms as well as a significant increase in functional AChR compared to untreated rats with EAMG as determined 6 to 10 weeks later [224].

Immunotoxins targeting AChR were recently proposed as an alternative to botulinum toxin, to induce a permanent correction of oculo-facial dystonias or some forms of ocular motility disorders.

Direct injection of the α-AChR IT ricin-mAb 35 into the extraocular muscles of rabbits resulted in a dose-related focal injury to the muscles, with a self-limited inflammatory component and significant muscle fiber loss. At the highest dose tested, there was substantial inflammatory cell infiltrate by 3 days, which largely disappeared by 7 days. Significant muscle loss was apparent by 7 days after treatment. Both the inflammatory reaction and muscle fiber loss were confined to the immediate injection site. At 14 days after treatment, early signs of muscle regeneration were evident within the tissue sections. No evidence of orbital or systemic toxicity was seen in any animal [225]. Extraocular muscles injection with ricin-mAb35 resulted in a sustained decrease in muscle mass at 105 days after injection, with subtler morphometric changes persisting even to 1 year [226]. Injection of ricin-mAb35 resulted in sustained weakness in extraocular muscle. Muscle force generation was assessed in rabbit at 1, 6, and 12 weeks after IT injection. Force generation declined in IT treated muscles at each postinjection interval. At 12 weeks, mean tetanic tension in treated muscles was reduced by 50% as compared with saline-injected controls. Single-twitch tension at 12 weeks was reduced by 33% as compared to controls. Similar effects were noted at 1 and 6 weeks. Fatigue rate was not greater in treated muscles at any post-injection intervals [227].

The mAb73-saporin immunotoxin injected into the extraocular muscles of rabbits caused focal damage, without histological changes in adjacent muscles. The histological examination revealed necrotic/apoptotic lesions restricted to the sites of inoculation and largely infiltrated by macrophages. No evident inflammatory reaction was detected at any time and neutrophils were substantially absent. At 14

days after injection, necrosis/apoptosis was still evident and the sclerotic reaction was minimal [228].

All together these results suggest that α-AChR ITs could be a more long-term alternative to botulinum toxin for the treatment of strabismus.

ABBREVIATIONS

α-	=	Anti-
AC_{50}	=	Concentration causing apoptosis in 50% of cells
ACh	=	Acetylcholine
ADCC	=	Antibody dependent cell cytotoxicity
AML	=	Acute myeloid leukaemia
APC	=	Antigen presenting cells
B-ALL	=	B-cell acute lymphoblastic leukaemia
B-CLL	=	B-cell chronic lymphocytic leukaemia
BFU	=	Burst forming unit
bR	=	Blocked ricin
BsAb	=	Bispecific antibody
CEA	=	Carcinoembryonic antigen
CFU	=	Colony forming unit
CFU-GEMM	=	Multipotent colony forming unit
CPA	=	Cyclophosphamide
CR	=	Complete remission
CSF	=	Colony stimulating factor
CTLA-4	=	Cytotoxic T lymphocyte antigen 4
dgA	=	Deglycosylated ricin toxin A-chain
EBV	=	Epstein-Barr virus
EFS	=	Event free survival
EGF	=	Epidermal growth factor
FGF	=	Fibroblast growth factor
GEL	=	Gelonin
GVHD	=	Graft versus host disease
HMW-MAA	=	High-molecular-weight melanoma-associated antigen
H-RS cells	=	Hodgkin and Reed-Sternberg cells
i.t.	=	Intra thecal
IC_{50}	=	Concentration inhibiting 50%
IT	=	Immunotoxin
LD_{50}	=	Lethal dose for 50% of animals
LTC-IC	=	Long-term culture initiating cells
mAb	=	Monoclonal antibody
MG	=	Myasthenia gravis
MLA	=	Mistletoe lectin A-chain
MLC	=	Mixed lymphocyte culture
MLR	=	Mixed lymphocyte reaction
MOM	=	Momordin
MST	=	Median survival time
MTS	=	Multicellular tumour spheroid
PAP	=	Pokeewed anti-viral protein
PAP-S	=	Pokeewed anti-viral protein from seeds
PBL	=	Peripheral blood lymphocytes
PBMC	=	Peripheral blood mononuclear cells
PMNC	=	Polymorphonuclear cells
PHA	=	Phytohemoagglutinin
PR	=	Partial remission
RIP	=	Ribosome-inactivating protein
RTA	=	Ricin toxin A-chain
SAP	=	Saporin-S6
scFv	=	Single chain fragment variable
SCID	=	Severe combined immunodeficiency disease
SMC	=	Smooth muscle cells
t_{10}	=	Time taken for a one log inhibition of protein synthesis compared with controls
$t^1/_2$	=	Half-life
T-ALL	=	T-cell acute lymphoblastic leukaemia
Tf	=	Transferrin
VEGF	=	Vascular endothelial growth factor.

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