

Chapter 2

Targeting the Transferrin Receptor to Overcome Resistance to Anti-Cancer Agents

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1 The Transferrin Receptor

The transferrin receptor (TfR) plays an important role in iron uptake and delivery [1]. The primary role of the TfR is to internalize iron through the binding of its natural ligand, the transferrin (Tf) protein that carries iron through the circulation. Iron is necessary for various cell processes such as respiration, metabolism, DNA synthesis and the proper functioning of various heme and nonheme proteins that require iron as a cofactor [1]. In addition, the TfR seems to be important for other processes such as cell growth and proliferation [1].

The TfR, a 180-kDa homodimeric glycoprotein, is a type II transmembrane receptor that has three important domains for its function (Fig. 2.1). It is composed of a C-terminal domain, also known as the ectodomain, a transmembrane region, and an N-terminal domain that is on the cytosolic side of the membrane. The ectodomain is important for binding to Tf for the internalization of iron. Two TfR genes have been identified, TfR1 and TfR2. Furthermore, the TfR2 gene produces two transcripts, α and β , that are produced by alternative splicing. TfR2 α shows similarity with TfR1 in that they exhibit a 45% similarity and 66% homology in their ectodomain. However, the cytoplasmic domains of the two proteins demonstrate no similarity [1]. The TfR2 β transcript lacks the transmembrane and cytoplasmic domains and its

function remains unknown. TfR1 and TfR2 α differ in cell surface expression and gene regulation. The TfR1 is ubiquitously expressed on normal cells at low levels. Increased TfR1 expression is observed on cells with a high proliferation rate, including cancer cells. TfR2 expression is limited to hepatocytes and enterocytes in the small intestine [1]. TfR2 expression has been found in some human cell lines such as B and myeloid cell lines as well as some cell lines derived from solid tumors [1]. TfR1 is post-transcriptionally regulated directly by intracellular iron levels as compared with TfR2 that is not. TfR2 is thought to be primarily regulated by the cell cycle and iron-bound Tf [1]. Thus both receptors differ considerably in expression and regulation indicating different roles in iron delivery. In addition, TfR1 has a 25-fold greater affinity for Tf relative to TfR2, indicating the main role of TfR1 in iron homeostasis [1].

Tf is an 80kDa monomeric glycoprotein composed of two lobes; an N and C lobe that are separated by a short spacer sequence (reviewed in [1]). Each lobe is capable of binding one iron molecule. The number of iron molecules bound to Tf has an important effect on the affinity of Tf for the TfR. At physiological conditions, holo Tf or diferric Tf (two iron) has the greatest affinity followed by monoferric (one iron), while apo-Tf (no iron) has the lowest affinity for the receptor [1]. Thus, iron uptake by the cell is mediated mostly through the

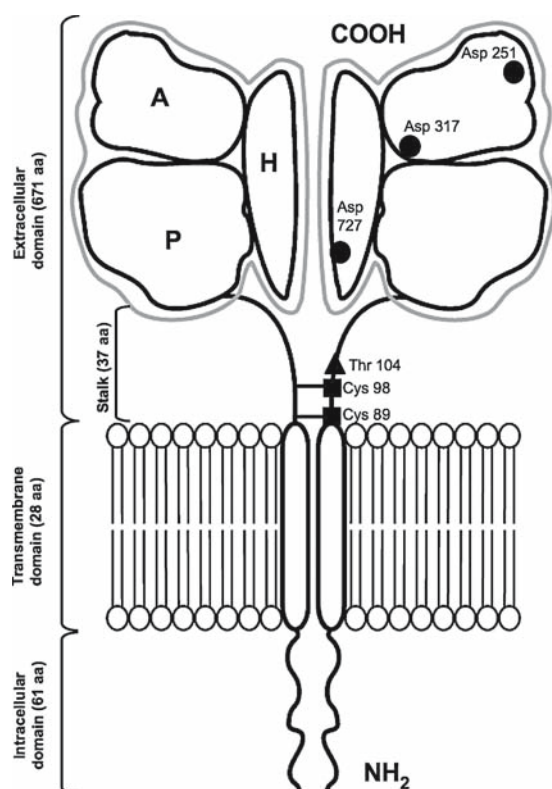


FIGURE 2.1. Schematic representation of the TfR. This receptor is a type II receptor found on the cell surface as a homodimer consisting of two monomers linked by disulfide bridges at cysteines 89 and 98 (■). The TfR contains an intracellular domain, a transmembrane domain, and a large extracellular domain. There is an O-linked glycosylation site at threonine 104 (▲) and three N-linked glycosylation sites on asparagine residues 251, 317, and 727 (●). The extracellular domain of the TfR consists of three subdomains: apical (A), helical (H) and protease-like domain (P). (Daniels et al. *Clinical Immunology* 2006, (121):144–158. Copyright 2006 Reprinted with the kind permission of Elsevier USA.)

interaction of diferric Tf and the TfR. The formation of a complex between diferric Tf and the TfR allows iron internalization into cells through a receptor-mediated endocytosis pathway (Fig. 2.2). This complex is internalized in a clathrin-coated pit into the cell and delivered into endosomes. Protons are pumped into the endosome causing an acidic change in the pH environment. This causes a conformational change in Tf that results in the release of iron. Iron can then be transported out of the endosome into the cytosol through a divalent metal

transporter (DMT1). The Tf/TfR complex remains inside the endosome until it is brought to the cell surface where apo-Tf dissociates from the TfR and is then free to circulate and bind free iron.

Many studies have used the TfR as a target for the delivery of various therapeutic agents (reviewed in [2]). The high expression of the TfR in cancer cells (that can be 10 to 100-fold greater than normal cells), its cell surface accessibility, and constitutive recycling pathway make this receptor an attractive target for immunotherapy. Importantly, either Tf or anti-TfR antibodies can mediate delivery of molecules by TfR targeting. The following is a discussion of the various strategies that have utilized targeting of the TfR (summarized in Fig. 2.3 and Table 2.1) to overcome cancer cell resistance to therapy or to provide the first hit in the “two hit signal” model to sensitize resistant cells to chemotherapeutic agents as combination treatment strategies. Both strategies are of great importance in treating patients whose cancers have developed resistance to common therapies and have thus developed more aggressive malignancies.

2 Tf Conjugates to Overcome Chemoresistance

Doxorubicin (Adriamycin®) (ADR) is an anthracycline chemotherapeutic drug used to treat a variety of cancers. ADR blocks DNA synthesis along with the activity of topoisomerase II, an enzyme that helps to relax the coil and extend the DNA molecule prior to DNA synthesis or RNA transcription. When used as a single treatment modality, ADR often exhibits devastating side effects including cardiotoxicity, myelosuppression, nephrotoxicity, and extravasation [3]. Systemic drug toxicity is often attributed to quick diffusion throughout the body resulting in a homogeneous tissue distribution [4]. The potential benefits of ADR treatment may also be blocked by the development of drug resistant cancer cells. ADR resistance can be attributed to many molecular events. Includes the overexpression of the multi-drug resistance (MRP) gene that codes for an active drug efflux pump P-glycoprotein on the cell surface that decreases cellular accumulation of the drug [5, 6]. ADR resistance may also be attributed to the impaired ability of drug trafficking or altered intracellular distribution within the cell

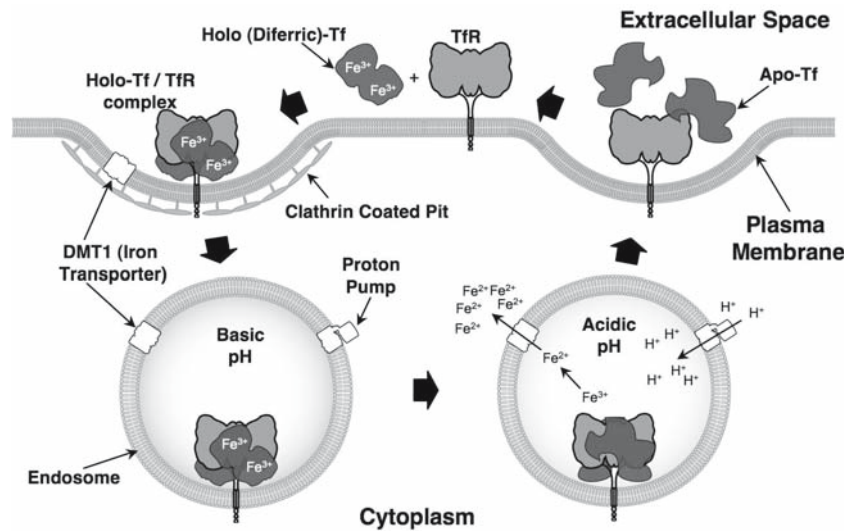


FIGURE 2.2. (See Color Plates.) Cellular uptake of iron through the Tf system via receptor-mediated endocytosis. The TfR consists of a dimer on the surface of the cell. Each receptor monomer binds one Tf molecule that consists of two lobes (the N and C lobes). Each lobe of Tf binds one iron molecule and thus two diferric Tf molecules bind to the receptor with high affinity. Endocytosis of the diferric Tf/TfR complex occurs via clathrin-coated pits and the complex is delivered into endosomes. Protons are pumped into the endosome resulting in a decrease in pH that stimulates a conformational change in Tf and its subsequent release of iron. The iron is then transported out of the endosome into the cytosol by the divalent metal transporter 1 (DMT1). Apo-Tf remains bound to the TfR while in the endosome and is only released once the complex reaches the cell surface. (Daniels et al. *Clinical Immunology* 2006, (121):144–158. Copyright 2006 Reprinted with the kind permission of Elsevier USA.)

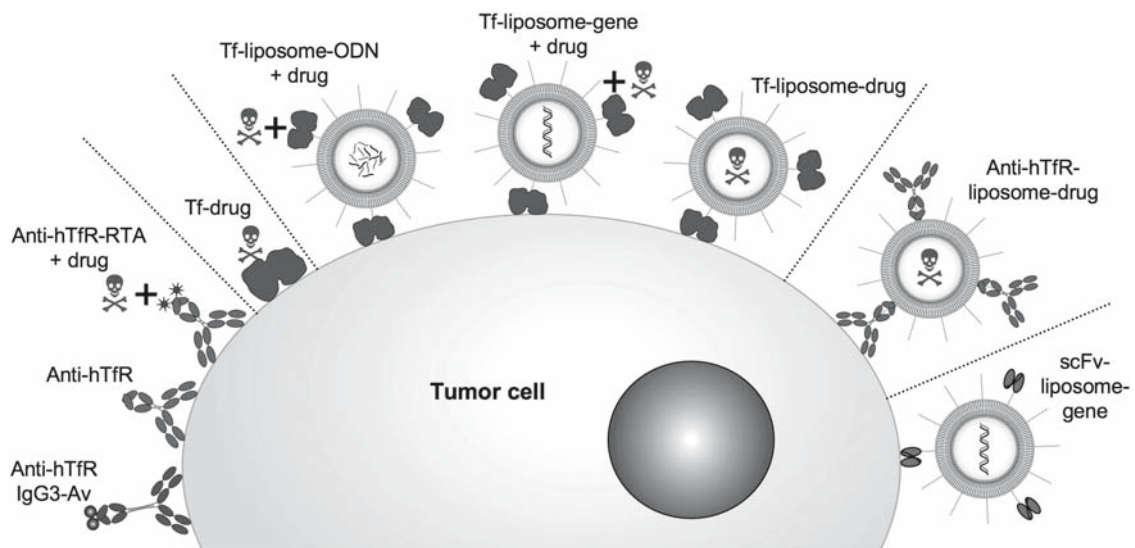


FIGURE 2.3. (See Color Plates.) Strategies for targeting the TfR to overcome resistance to chemotherapy. The TfR can be targeted by direct interaction with conjugates of its ligand Tf as well as by monoclonal antibodies or antibody conjugates that target the extracellular domain of the TfR. Targeting the TfR has been utilized to deliver chemotherapeutic drugs, protein toxins, and liposomes containing either drugs, antisense oligonucleotides, or genes into malignant cells in order to sensitize resistant cells to chemotherapy. Adapted from (Daniels et al. *Clinical Immunology* 2006, (121):144–158. Copyright 2006 Reprinted with the kind permission of Elsevier USA.)

TABLE 2.1. Targeting the TfR to overcome resistance or to sensitize cancer cells to chemotherapy.

Conjugate	Cell model	Comments	References
Tf conjugates			
Tf-ADR	K562 erythroleukemia	3-fold Increase in cytotoxicity compared with free ADR; Restored IC_{50} of ADR-resistant cells to that of sensitive cells	11
	HL-60 leukemia	10-fold Increase in sensitivity in ADR-resistant cells	12
	Lovo colorectal carcinoma	5- to 10-fold increase in sensitivity in ADR-resistant cells	11, 13
	Hep2 laryngeal carcinoma		13
	H-MESO-1 mesothelioma	5- to 10-fold increase in sensitivity in ADR-resistant cells; Prolonged the life span in a xenograft model	13
Tf-GN	KB cervical cancer	Decreased IC_{50} 4-40 fold (depending on the cell line)	14
	CCRF-CEM T-lymphoma	Cytotoxicity enhanced by Bortezomib	16
	MCF-7/ADR	Decreased IC_{50} 100-fold	17
Tf-Liposome conjugates			
Tf-Liposome-ADR	MXT-2B metastatic mammary cancer	2-fold Increase in cytotoxicity compared with nontargeted liposome; 2.4-fold increase compared with ADR alone	18
Tf-liposome-cisplatin	MKN45 Gastric cancer	Increase in survival rate in a xenograft model	20
Tf-liposome-hoFR ODN	MDA-MB-435 Breast cancer	Increased cytotoxicity 10-fold compared with ADR alone; Sensitized cells to ADR treatment	21
Tf-liposome-Bcl-2 ODN	K562 Erythroleukemia	Decreased Bcl-2 expression 10-fold compared with ODN alone; Sensitized cells to daunorubicin (10-fold increase in cytotoxicity)	25
	MCF-7 Breast cancer	Increase in apoptosis, cytochrome c release, and caspase-8 activation	26
Tf-liposome-p53	JSQ-3 Head and neck cancer	Sensitization to radiotherapy (dependent on level of restored p53 expression); Complete tumor regression and no recurrences for up to 6 months in a xenograft model	29
Antibody-liposome conjugates			
OKT9-liposome-ADR	K562/ADM	Murine anti-hTfR IgG1; Decreased IC_{50} 3.5 fold compared with free ADR; Restored intracellular levels of ADR in resistant cells	19
ScFv-liposome-p53	MDA-MB-435 Breast cancer	Increased p53 expression 10-fold	28
	DUI45 Prostate cancer		
	JSQ-3 Head and neck cancer	Systemic delivery increased p53 expression in subcutaneous tumors in a xenograft model	28
	435/LCC6 Breast cancer	Combination treatment with docetaxel prolonged survival in a xenograft model	28

Antibody or antibody conjugates		
A24 + chemotherapy	Activated PBMC from normal or acute T-cell leukemia/lymphoma	30-31
	Murine IgG2b anti-hTfR; proapoptotic by itself; Competes with Tf and blocks iron uptake; Decreases TfR surface expression; Enhanced cytotoxicity observed with combined treatment with chemotherapy	
7D3-RTA + ADR	H-MESO-1 Mesothelioma	33
	Murine IgG1 anti-hTfR; no effects when used alone; 7D3-RTA increased cytotoxicity; 7D3-RTA+ADR no additive effects observed in vitro, but enhanced median survival in a xenograft model	
454A12-RTA + rhIFN	OVCAR-3 Ovarian cancer	35
	Murine IgG1 anti-hTfR; no effects when used alone; No effect of rhIFN alone; control median survival = 41 days; 454A12-RTA increased survival to 89 days; 454A12-RTA+rhIFN increased survival to >120 days; Tumor burden dependent; Chemotherapy decreased tumor burden prior to treatment	
Antibody fusion proteins		
Anti-rat TfR IgG3-Av	Y3-Ag 1.2.3 rat myeloma C58 (NT) D.1.G.OVAR.1 rat T-cell lymphoma	36, 40
	proapoptotic alone; Delivers active biotinylated agents into tumor cells	
Anti-human TfR IgG3-Av	Malignant B-cell lines (ARH-77 and IM-9) and primary CD138+ cells isolated from patients (one diagnosed with multiple myeloma and one with plasma cell leukemia)	36, 37
	Induced rapid degradation of TfR; Cross-linking of TfR is important for cytotoxicity; Induced iron-dependent apoptosis	

rather than its accumulation inside the cell [7, 8]. It has been shown that chemotherapeutic drugs are distributed throughout the cytoplasm and nuclei of sensitive cells. In resistant cells, the localization of these drugs changes and they are only found within discrete cytoplasmic organelles [9, 10]. Thus, drug localization within the tumor cell is important for therapeutic efficacy.

In an attempt to overcome these problems, delivery of ADR into the tumor through targeted therapy via the TfR has been widely studied (reviewed in [2]). Conjugation of Tf to ADR has been effective in overcoming drug resistance in a variety of malignant cell lines. In fact, ADR resistant sublines of K562 human erythroleukemic cells and HL-60 human promyelocytic leukemia cells exhibited enhanced sensitivity to Tf-ADR relative to the use of ADR alone. Resistant K562 and HL-60 cells were 3- and 10-fold more sensitive to the conjugate than ADR alone, respectively [11]. Another study demonstrated that the K562 parental cell line had an IC_{50} of 3.2 nM with ADR alone compared with 20 nM for the resistant subline [12]. Treatment of the resistant subline with Tf-ADR induced potent cytotoxic effects with IC_{50} of 3.6 nM, which was comparable to that of the K562 parental (sensitive) cell line.

Other studies with the resistant sublines of Lovo (human colon carcinoma), H-MESO-1 (human mesothelioma), Hep2 (human laryngeal carcinoma cells), and HL-60 (human promyelocytic leukemia) also exhibited cytotoxicity with Tf-ADR treatment [13]. More importantly, the conjugate showed greater potency toward resistant cell lines relative to sensitive cell lines. In ADR resistant cells a 5- to 10-fold increase in cytotoxicity was observed with Tf-ADR treatment relative to ADR alone. ADR-sensitive cells exhibited a smaller increase in cytotoxicity when treated with Tf-ADR (4- to 5-fold more toxic than free ADR) [13]. Multidrug resistant (MDR) KB (human cervical cancer) also exhibited significant cytotoxicity to the Tf-ADR conjugate [14]. Toxicity of the Tf-ADR conjugate was observed in MDR KB cell lines; KB-8-5 (partially MDR) and the highly MDR cells KB-C1 and KB-V1. Treatment with the conjugate in all KB MDR cells exhibited greater cytotoxicity and a lower IC_{50} than ADR treatment alone. In vivo studies with nude mice bearing the H-MESO-1 cell line prolonged the life span of the treated mice

compared with ADR alone or unlinked ADR and Tf [13]. Treatment with the conjugate increased the life span of mice from 39% in mice treated with ADR alone, to 69% in mice treated with the Tf-ADR conjugate. Even though there were no long-term survivors, tumor burden was significantly decreased in conjugate treated mice relative to ADR treatment alone [13].

A Tf conjugate consisting of the anti-neoplastic metallo-drug gallium nitrate (GN) has also been targeted by conjugates of Tf in order to sensitize cancer cells to common therapeutics. GN shares chemical characteristics with iron and binds free Tf in human blood (reviewed in [15]). The Tf-GN complexes enter cells through TfR-mediated endocytosis. In this way, GN blocks cellular iron uptake and induces a lethal iron deprivation within the cell. GN also has direct effects on cells, such as inhibition of DNA polymerases, membrane tyrosine phosphatase activity, magnesium-dependent ATPase, and tubulin polymerization. However, the extent to which these mechanisms contribute to the anti-cancer effects of GN are not clear. The final result of GN treatment is the induction of apoptosis through a mitochondrial-mediated pathway. GN alone is commonly used for the treatment of non-Hodgkin's lymphoma [15]. However, this activity was recently shown to be enhanced by the proteasome inhibitor Bortezomib in the CCRF-CEM human T-lymphoma cell line suggesting the efficacy of the combination treatment with the Tf-GN conjugate [16]. Furthermore, a Tf-GN-ADR conjugate showed cytotoxicity in an MDR cell line MCF-7/ADR (human breast cancer cell line resistant to ADR) [17]. This study indicated that the MCF-7/ADR was 1,000-fold more resistant than the parental cell line MCF-7 to free ADR treatment. Tf-GN-ADR exhibited the same inhibitory effect as ADR alone in MCF-7. However, in the resistant cell line MCF-7/ADR, treatment with the Tf-GN-ADR conjugate decreased the IC_{50} by 100-fold [17]. Another conjugate consisting of Tf, iron, and ADR (Tf-Fe-ADR) also demonstrated enhanced cytotoxicity, but only by 10-fold. Tf-GN-ADR was also compared with Tf-GN alone and was found to be 500- and 3,000-fold more cytotoxic to MCF-7 and MCF-7/ADR, respectively. This study indicates that the Tf-GN-ADR conjugates reversed ADR resistance that was accompanied by a decrease in the MRP expression

[17]. Drug resistance in cancer patients introduces various complications in treating relapsed and disseminated malignancies. These studies indicate that the Tf-ADR or Tf-GN-ADR conjugates can be effectively used as sensitizing agents to induce cytotoxic effects in ADR-resistant or MDR cell lines. Furthermore, these conjugates have the potential to be utilized to treat patients who have developed MDR for the effective treatment of cancer malignancies.

3 TfR Targeted Liposomal Delivery of Chemotherapeutic Drugs

The delivery of ADR through the targeting of the TfR by a Tf-liposomal system may also be a successful therapeutic approach. Efficacy of this system has been shown in vitro in MXT-B2, a human metastatic mammary carcinoma cell line [18]. The Tf conjugated liposomes encapsulating ADR enhanced cytotoxicity about 2-fold compared with a nontargeted liposome carrying ADR and 2.4-fold compared with free ADR in MXT-B2 cells. The anti-human TfR (hTfR) antibody OKT9 (IgG1) has also been used to deliver liposomes encapsulating ADR into the resistant human erythroleukemic cell line K562/ADM [19]. In this cell line, the targeted liposomal delivery of ADR decreased the IC_{50} 3.5-fold compared with free ADR. Interestingly, the IC_{50} of the targeted liposome was similar to free ADR in the parental, ADR-sensitive K562 cell line. This study also showed that the intracellular level of ADR in the K562/ADM (resistant) cells rapidly decreased (due to drug efflux) after free ADR treatment and was significantly lower (15- to 45-fold) compared with the parental K562 cell line, which maintained high levels of intracellular ADR. However, resistant cells treated with the OKT9-ADR-liposomes maintained similar levels of intracellular ADR compared with sensitive cells. This suggests that delivery of ADR through the TfR results in decreased ADR efflux, resulting in higher intracellular accumulation and cytotoxic effects. How the TfR is involved in the blockage of ADR efflux remains elusive; however, the route

of delivery through TfR mediated endocytosis is expected to play a key role in altering the trafficking of ADR so that it is retained within the resistant tumor cell [7, 8].

The efficient delivery of cisplatin, a platinum-containing chemotherapeutic drug that forms cytotoxic adducts with the DNA, into cells has also been achieved through Tf-liposomal therapy [20]. This system utilizes polyethylene glycol (PEG) to avoid uptake by the reticuloendothelial system and thus increases the circulation time of the liposomes. Tf is then covalently linked to PEG to form a liposome that targets the TfR on tumor cells. This system efficiently delivered cisplatin in peritoneal dissemination xenografts of MKN45, a human gastric cancer cell line, in BALB/cA-JcI-nu nude mice. An increase in the survival rate was observed in mice treated with this targeted liposomal system (40% of mice survived after 60 days of treatment) as compared with control animals (21-day survival), PEG liposomes (50-day survival), and cisplatin alone (45-day survival). More importantly, all survivors treated with the targeted liposomes were tumor free after the 60-day period. Tf-liposome internalization into cancer cells was greater than nontargeted liposomes or PEG alone, indicating the importance of targeting the TfR. Also uptake by the spleen and liver was significantly lower than nontargeted liposomes further demonstrating the specific delivery of cisplatin to tumor cells.

4 TfR Targeted Liposomal Gene Therapy to Sensitize Cells to Chemotherapy

Targeted liposomes can also be used for gene therapy purposes to sensitize cells to chemotherapy. This strategy can be used to deliver antisense oligodeoxynucleotides (ODN) to cells in order to knock down expression of tumor-promoting proteins. In addition, this technique can also be used to delivery entire genes into cells in order to restore expression of antitumor proteins whose expression has been lost during malignant transformation. Both possibilities are further discussed in the following sections.

4.1 Human α Folate Receptor Oligodeoxyribonucleotide Delivery

The efficacy of Tf-liposomes to deliver antisense nucleotides complementary to the human α folate receptor gene sequence (h α FR) through the TfR has been demonstrated [21]. The h α FR is a surface glycoprotein that has high affinity for folic acid and reduced folates that promotes cell proliferation. This receptor is highly upregulated on cancer cells [22] and thus promotes the growth of the tumor. Tf-mediated delivery of ODN to knock down h α FR expression in a panel of human breast carcinoma cell lines significantly decreased cell survival [21]. It was also demonstrated that targeted delivery of the h α FR ODN sensitizes MDA-MB-435 human breast cancer cells to ADR treatment. In fact, cell death was increased by 5-fold compared with delivery of the sense ODN control and 10-fold compared with ADR alone treated cells.

4.2 Bcl-2 Oligodeoxyribonucleotide Delivery

The upregulation of Bcl-2, an anti-apoptotic protein in various malignant cells, is often associated with the development of chemoresistance [23], including resistance to daunorubicin (Cerubidine[®]) an anthracycline topoisomerase inhibitor that blocks DNA synthesis and RNA transcription [24]. In order to block this up-regulation and overcome chemoresistance, Bcl-2 ODN have been delivered into tumor cells via Tf conjugated liposomes [25]. Efficient uptake of these liposomes in K562 human erythroleukemia cells was observed compared with nontargeted liposomes and could be blocked by the addition of free Tf. Bcl-2 expression was down-regulated 2-fold compared with the nontargeted liposomes and 10-fold relative to the Bcl-2 ODN alone.

Tf targeted delivery of Bcl-2 ODN sensitized cells to chemotherapy as demonstrated by a 10-fold increase in toxicity observed with the combination treatment compared with nontargeted liposomal delivery or daunorubicin alone. Tf-liposomes have also been utilized to deliver Bcl-2 ODN into breast cancer cells as a chemosensitizing agent. Synergistic effects were observed in MCF-7 breast cancer cells with the combination treatment of Tf-Bcl-2 ODN liposomes and cisplatin [26]. Cells

containing the p53 tumor suppressor protein (see following section) as well as those lacking p53 expression were found to be sensitive to the combination treatment [26]. Thus, this treatment strategy may be beneficial irrespective of the p53 status of the cell. These studies demonstrate the efficacy of using Tf targeted liposomes as chemosensitizing agents to overcome resistance to single treatment strategies.

4.3 Wild-Type p53 Gene Delivery

The tumor suppressor protein p53 is a transcription factor that is activated upon DNA damage and has been termed the “guardian of the genome” [27]. Activation of p53 leads to cell cycle arrest, DNA repair, and or even cell death if the DNA damage is too extensive. p53 is mutated in many types of malignancies and this loss of function results in genomic instability and impaired apoptosis. The reintroduction of the wild-type p53 gene into malignant cells has been a common goal for many gene therapy strategies.

Single chain antibodies (scFv) targeting the TfR have also been used as delivery vehicles complexed to liposomes for targeted gene delivery. One such anti-TfR scFv contains the single chain variable region of the murine IgG1 anti-hTfR 5E9 antibody conjugated to the surface of liposomes. This immunolipoplex successfully delivered the p53 tumor suppressor gene both in vitro and in vivo [28]. In vitro p53 gene delivery was increased 10-fold in MDA-MB-435 human breast cancer cells and DU145 human prostate cancer cells. In a mouse xenograft model of the human squamous cell carcinoma of the head and neck using the JSQ-3 (containing mutated p53) cell line, systemic delivery of the 5E9 scFv-targeted liposomes efficiently delivered the p53 gene to tumor cells that were implanted subcutaneously and also resulted in high expression of the wild-type p53 protein [28]. Much lower p53 expression was observed in mice treated with a nontargeted liposomes containing the p53 gene. A xenograft model of breast cancer metastasis (using the 435/LCC6 cell line derived from mouse ascites of breast cancer cell line MDA-MB-435) was used to determine the in vivo efficacy of the 5E9 scFv targeted liposomes to sensitize cells to treatment with the microtubule stabilizer docetaxel [28]. No antitumor efficacy

was observed with docetaxel treatment alone, which suggests that this tumor model is highly chemoresistant. Systemic administration of 5E9-liposomes containing the p53 gene in combination with docetaxel significantly prolonged survival (60–80% long term survival) compared with either agent alone. Importantly, no general toxicity was observed in any of the treated mice. The antitumor activity was equivalent to Tf-liposomes containing the p53 gene tested in the same study. Altogether this study demonstrates that delivery of wild-type p53 to tumor cells successfully sensitizes them to chemotherapy and demonstrated antitumor effects at lower chemotherapeutic doses. This strategy could also potentially decrease the severity of docetaxel-induced side effects. Ultimately this study demonstrates that targeting of the TfR is beneficial for the systemic delivery and restorative expression of wild type p53 as a chemosensitizing agent.

In vitro studies have shown that high levels of wild-type p53 expression can also be obtained using liposomes conjugated to Tf [29]. These studies were performed in the human head and neck cell line JSQ-3 that was derived from a radioresistant tumor of the nasal vestibule. Restoration of p53 expression in these cells sensitized them to radiation treatment, which was dependent on the level of wild-type p53 expression. In vivo efficacy was also demonstrated in a xenograft mouse model [29]. JSQ-3 tumors were established subcutaneously in athymic nude mice. After 7–10 days of tumor growth, mice were treated systemically with Tf-p53-liposomes twice a week for a total of 5 injections. Radiotherapy at the tumor site began 48 hours after the first injection of the Tf-p53-liposomes and was given every 48 hours for a total of 10 treatments. This treatment strategy resulted in complete tumor regression and blocked recurrence for up to 6 months.

5 Anti-TfR antibodies and Their Conjugates For Sensitization of Tumors to Chemotherapy

Another strategy to overcome the resistance to common anticancer therapies is to use anti-human TfR (hTfR) antibodies alone (if they interfere with TfR function) or conjugates made with these anti-

bodies in combination treatment strategies with chemotherapeutic drugs. For example, the murine anti-hTfR IgG2b antibody A24 has been used for this purpose. A24 competes with Tf for TfR binding that leads to a block in iron uptake, impaired cycling of the TfR, and decreased surface expression of the receptor [30, 31]. A24 alone has anti-proliferative and proapoptotic against peripheral blood mononuclear cells (PBMC) activated by phytohemagglutinin (PHA)/IL-2 or cocultured with activated dendritic cells. PBMC activated T cells isolated from both normal donor and patients with adult T-cell leukemia/lymphoma (ATL) were sensitive to the effects of the A24 antibody. Enhanced cytotoxic effects were observed when tumor cells isolated from a patient with chronic ATL were treated with a combination of the A24 antibody and chemotherapy (either the nucleoside reverse transcriptase inhibitor zidovudine (AZT), the cytokine interferon- α , or the topoisomerase II inhibitor VP-16) [31].

Some anti-hTfR antibodies do not have cytotoxic effects when used as a single treatment. For example, the murine anti-hTfR IgG1 antibody 7D3 has no cytotoxic effects when used alone. However, when used as a component of an immunotoxin (a cell-specific ligand linked to a plant or bacterial toxin or modified toxin subunit) [32] its antitumor effects can be greatly enhanced [33]. The ricin toxin, derived from the seed of the *Ricinus communis* plant, is a ribosomal inactivating protein that is composed of two chains connected by a disulfide bond [34]. The A chain of ricin (RTA) contains the N-glycosidase enzyme that blocks ribosomal activity, while the B chain (RTB) is responsible for binding to the cell surface. RTA has no cytotoxic effects by itself because it can not enter the cells and has been used for the development of potent hTfR targeted immunotoxins (reviewed in [2]). 7D3 when chemically conjugated to RTA demonstrated significant antitumor effects in H-MESO-1, a human mesothelioma cell line [33]. These effects of the 7D3-RTA conjugate have been observed both in vitro and in vivo in a xenograft mouse model. In vitro analysis of the combination treatment of 7D3-RTA and ADR showed no synergistic effects of the combination treatment. However, in vivo studies demonstrated that this combination treatment enhanced the median survival to 31 days compared with 22 days for ADR alone and 23 days

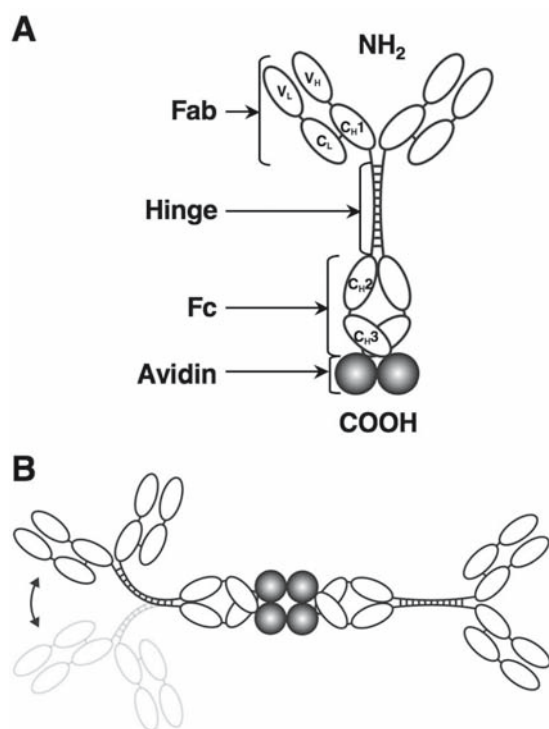


FIGURE 2.4. Schematic representation of the structure of anti-TfR IgG3-Av. A) This fusion protein consists of a murine/human chimeric antibody specific for either rTfR or hTfR. Each fusion protein has one avidin molecule genetically fused to each C_H3 domains of the human IgG3 Fc region. B) FPLC analysis suggest that anti-TfR IgG3-Av exists as a dimer in solution [36]. The extended hinge region of the IgG3 molecule allows for increased flexibility of the fusion protein.

for the 7D3-RTA conjugate alone [33]. The addition of a third component, the sodium ionophore monensin, further increased survival to 41 days. Thus, the combination of 7D3-RTA with chemotherapy demonstrates synergistic antitumor effects that could be used for the treatment of solid tumors in humans.

The murine anti-hTfR IgG1 (454A12) antibody alone also is not an effective treatment. However, an 454A12-RTA conjugate has shown synergistic effects with standard therapeutic regimes. The efficacy of the conjugated to RTA was studied in a human ovarian carcinoma xenograft model [35]. Ten days after intraperitoneal injection of OVCAR-3 cells, mice were treated with the 454A12-RTA immunotoxin alone or in combination with recom-

binant human interferon- α (rhuIFN). Treatment with rhuIFN alone demonstrated no antitumor effects. Mice treated with 454A12-RTA alone showed an increased median survival time (89 days) compared with the saline control group (41 days). Combination treatment of 454A12-RTA and rhuIFN further increased the survival time to more than 120 days. However, if treatment was initiated when the tumor burden was higher (15 days after tumor injection) no additive effect was observed with the 454A12-RTA and rhuIFN treatment, indicating that tumor burden plays a role in determining antitumor efficacy of the combination treatment. The pretreatment of animals with large tumor burdens with chemotherapy, either cyclophosphamide (a nitrogen mustard that cross-links DNA) or cisplatin, to reduce tumor volume prior to treatment significantly enhanced the antitumor effects of 454A12-RTA and rhuIFN combined therapy. After chemotherapy, mice treated with rhuIFN alone showed a significant increase in survival (89 days) compared with control mice (77 days). 454A12-RTA alone increased survival to 129 days while the combination of 454A12-RTA and rhuIFN increased survival to 162 days [35]. This study clearly demonstrates the benefit of using multicomponent treatment strategies as cancer therapeutics.

6 A Universal Delivery System for Targeted Cancer Therapy

We have previously constructed two chimeric antibody fusion proteins that target either the rat TfR (rTfR) or the human TfR (hTfR) [36, 37]. Both antibodies contain human IgG3 constant regions and have chicken avidin (Av) genetically fused to the C_H3 domains (Fig. 2.4). Anti-rTfR IgG3-Av contains the variable regions of the murine monoclonal antibody OX26 while anti-hTfR IgG3-Av is composed of the variable regions of the murine antibody 128.1. These antibody fusion proteins are the first anti-TfR of its kind, since IgG1 and IgG2 antibodies have been more commonly used for therapeutic purposes. The rationale behind utilizing IgG3 is due to the presence of an extended hinge that may facilitate simultaneous binding to antigen and Fc receptors to maximize its therapeutic potential. Both antibodies were developed to function as universal vectors for the delivery

of biotinylated agents into malignant cells that overexpress TfR. Unexpectedly, we have found that both of these antibodies alone are capable of inducing significant antiproliferative/proapoptotic effects in malignant cells, including cells that have developed resistance to various therapeutic drugs. Dimerization of these fusion proteins was shown to be important for this cytotoxicity. FPLC analysis showed that the anti-rTfR IgG3-Av exists as a dimer in solution [36]. Avidin is a tetramer comprised of four noncovalent monomers. Since each anti-hTfR IgG3-Av molecule contains two avidin moieties, dimerization may be caused by the formation of the tetrameric form of avidin (see Fig. 2.4). This suggests that cross-linking of the TfR due to the tetravalency of anti-hTfR IgG3-Av

is responsible, at least in part, for the cytotoxicity of the fusion protein. However, we can rule out the possibility that avidin (a positively charged molecule with heparin-binding ability) [38], the extended hinge region of human IgG3 [39], and/or the binding of the Fc fragment of IgG3 to Fc receptors may contribute to cross-linking induced by anti-hTfR IgG3-Av.

The efficacy of anti-rTfR IgG3-Av as a universal vector for targeted delivery of biotinylated agents has been demonstrated [36]. The avidin moiety of this fusion protein forms strong noncovalent interactions with biotinylated molecules such as glucose oxidase and β -galactosidase. Studies of this antibody fusion protein complexed to biotinylated-FITC indicate that the complex is internalized through

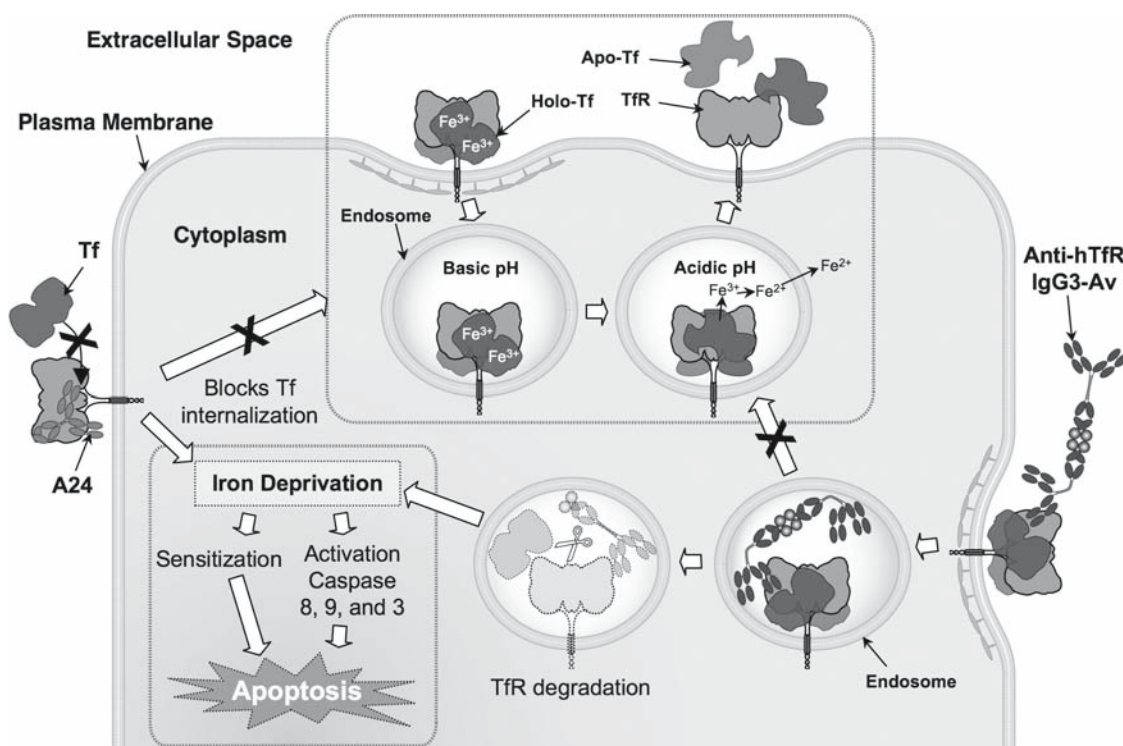


FIGURE 2.5. Schematic representation of the proposed mechanism of cytotoxicity of anti-hTfR antibodies. Under normal conditions (*top box*), diferric transferrin binds the TfR on the surface of the cell and is internalized via a clathrin-coated pit into an endosome. Due to the decrease in pH, iron dissociates from Tf and is pumped out of the endosome. The Tf/TfR complex traffics back to the cell surface where Tf dissociates from its receptor. Treatment with the A24 anti-TfR antibody (*left side of the figure*) blocks the binding of Tf and thus leads to iron deprivation, apoptosis and/or chemosensitization. Anti-hTfR IgG3-Av treatment (*right side*) disrupts the normal cycling pathway leading to the degradation of the TfR. Iron deprivation results are lethal to some malignant B cells. In cells that are resistant to anti-hTfR IgG3-Av cytotoxicity, the treatment could be used to sensitize cells to chemotherapy due to induction of iron starvation. A24 has also been shown to disrupt TfR cycling leading to a reduction of cell surface TfR expression. This may also contribute to the cytotoxic activity of A24. (See Color Plates)

receptor-mediated endocytosis [36]. The antibody fusion protein was also found to effectively deliver large biotinylated proteins (β -galactosidase, 464kDa) into malignant cells that retained activity in the intracellular environment. The anti-rTfR IgG3-Av exhibited antiproliferative/proapoptotic effects in rat cell lines of hematopoietic origin that have developed resistance to therapeutic agents. Anti-rTfR IgG3-Av exhibited these effects in Y3-Ag 1.2.3, a rat myeloma cell line resistant to 8-azaguanine (a purine analog interferes with purine biosynthesis), and C58 (NT) D.1.G.OVAR.1, a rat T-cell lymphoma resistant to 6-thioguanine (an antimetabolite cancer drug that interferes with purine biosynthesis) and ouabain (a poisonous glycoside that blocks the sodium potassium ATPase). These studies indicate that anti-rat IgG3-Av has dual activity through direct cytotoxic activity and the delivery of active biotinylated molecules.

Anti-rTfR IgG3-Av has also been used for antibody-directed enzyme prodrug therapy (ADEPT) [40]. ADEPT works in a two-step manner, the first is the administration of the antibody fusion protein conjugated to a prodrug processing enzyme in order to deliver the enzyme to the tumor cell. In the second step, a prodrug is administered in a nontoxic form following antibody/enzyme treatment and is metabolized by the tumor-localized enzyme. This form of therapy works to potentially eliminate nonspecific effects in normal cells that are caused by the use of chemotherapeutic agents alone. This form of therapy also has potential for eliminating minimal residual disease associated with treating solid tumors. As previously discussed, anti-rTfR IgG3-Av has been shown to efficiently deliver biotinylated proteins into cells, however limitations associated with binding variability can occur due to the presence of various biotinylated sites within the protein. Fusion of P67, the carboxy-terminal domain of human propionyl-CoA carboxylase α subunit, to a protein can eliminate this variability since P67 allows biotinylation at only one residue (lysine-669) by human or *E. coli* biotin protein ligases. In these studies, P67 was genetically fused to FCU1, a genetically engineered chimeric protein consisting of cytosine deaminase (CD) and uracil phosphoribosyltransferase (UPRT). CD converts the nonactive and nontoxic prodrug 5-fluorocytosine (5-FC) to a highly toxic 5-fluorouracil (5-FU). UPRT can then convert 5-FU to the toxic metabolite

5-fluorouridine 5'-monophosphate (5-FUMP). P67 was also fused to an *E. coli* enzyme PNP (purine nucleoside phosphorylase). PNP works by cleaving the prodrug 2-fluoro-2'-dexoyadenosine (F-dAdo) to produce the highly toxic 2-fluoroadenine (F-Ade). These two fusion proteins were then conjugated to anti-rTfR IgG3-Av after monobiotinylation of P67. Efficient monobiotinylation of P67 (>95%) was achieved and the prodrug processing enzymes retained their activity in the antibody fusion protein/enzyme complex. The antigen binding capability of the antibody was also preserved allowing successful targeting of the tumor cells. Anti-rTfR-Av effectively localized FCU1 and PNP to tumor cells so that the pro-drugs were metabolized into their cytotoxic forms. The prodrugs alone had cytotoxic effects at high concentrations, however, cytotoxicity increased by 50-fold when the cells were pretreated with either anti-rTfR-Av/P67-FCU1 or anti-rTfR-Av/P67-PNP in Y3-Ag1.2.3 rat myeloma cells [40]. These cells were found to be more susceptible to F-dAdo treatment. No cytotoxic effects were observed for a nontargeted complex. Importantly, this study supports the use of this antibody fusion protein as a delivery system for biotinylated therapeutic agents into tumor cells and its possible use as a sensitizing agent in resistant tumor cells.

Like its rat counterpart, the anti-hTfR IgG3-Av fusion protein has also demonstrated intrinsic cytotoxicity in human malignant cells. This was indicated by studies in the human erythroleukemia cell line K562, a panel of human malignant B-cell lines as well as malignant cells isolated from patients with multiple myeloma and plasma cell leukemia [36, 37]. ARH-77 and IM-9, Epstein-Barr virus (EBV) transformed lymphoblastoid cell lines, were found to be most sensitive to anti-hTfR IgG3-Av treatment [37]. Different levels of sensitivity to anti-hTfR IgG3-Av were observed for other malignant B-cell lines. More importantly, the fusion protein exhibited anti-proliferative effects in 8226/DOX40, a doxorubicin resistant variant of 8226/S, indicating that this antibody was capable of inducing cytotoxic effects in chemoresistant cells.

The mechanism of anti-hTfR IgG3-Av cytotoxicity has recently been explored (see Fig. 2.5) [37]. Upon treatment with anti-hTfR IgG3-Av, the TfR is rapidly degraded. Confocal microscopy indicated that the TfR colocalized with an intracellular compartment expressing LAMP-1 within 15 minutes of

treatment. This indicated that trafficking is altered and that the TfR is directed to a lysosomal compartment. Cells treated with anti-hTfR IgG3-Av indicated the presence of small TfR fragments that appeared to be the result of proteolytic cleavage, suggesting that degradation may take place in or prior to the LAMP-1 intracellular compartment. Degradation of the TfR was partially blocked by a cysteine protease inhibitor indicating that a papain-like cysteine protease is involved in the degradation of the N-terminus of the receptor. Other protease inhibitor treatments tested did not completely block degradation, suggesting other protease families may also be involved. Treatment with chloroquine or ammonium chloride showed that the degradation of the receptor was not sensitive to changes in the pH of the lysosome, indicating that the proteases involved in TfR degradation were still active and functional under these conditions.

Iron is important for dividing cells and its absence has been shown to cause mitochondrial-mediated cell death [41]. In two highly sensitive human B-cell lines (IM-9 and ARH-77), anti-hTfR IgG3-Av causes iron deprivation in cells leading to mitochondria depolarization and activation of caspase 9, 8, and 3 [37]. These cytotoxic effects are blocked by iron supplementation indicating the role of iron deprivation in inducing such effects. Treatment of anti-hTfR IgG3-Av and a pan-caspase inhibitor, Z-VAD-FMK, only partially blocked cell death, suggesting cell death is not solely dependent on the caspase activation pathway, but that other mechanisms may be involved. In addition, cells treated with the antibody fusion protein and Z-VAD-FMK still exhibited loss of mitochondrial potential and eventually died. Thus, anti-hTfR IgG3-Av induced lethal iron deprivation that can lead to mitochondrial-mediated apoptosis and chemosensitization.

These studies support the use of this fusion protein as an effective therapeutic agent that can induce significant cytotoxic effects in addition to its ability to deliver biotinylated molecules into cancer cells. Anti-hTfR IgG3-Av may also be used in combination treatment strategies with nonbiotinylated agents (such as chemotherapeutic drugs) to sensitize resistant cells to chemotherapy. This antibody fusion protein has the potential to be used as a universal delivery vector for the treatment of human malignancies and as an effective sensitizing agent for the "two-hit signal" model.

7 Conclusion

Upregulation of the TfR on the surface of malignant cells and its constitutive internalization pathway make this an attractive molecule for targeted cancer therapy. The main advantage of targeting the TfR is the versatility of both the targeting strategy itself as well as the agent to be delivered. The TfR can be targeted by its ligand Tf, antibodies, or antibody fragments specific for its extracellular domain. In addition, delivery of a therapeutic agent that targets a defect in cancer cells, like the restoration of wild-type p53 expression, further enhances tumor specificity without affecting normal cells. In these two ways, targeting the TfR would help to minimize the nonspecific effects of the treatment by more adequately targeting the malignant cells. Furthermore, it can be successfully used for the treatment of chemoresistant tumors and has served to sensitize cancer cells to cell death induced by chemotherapy, either by directly inducing iron starvation or by the delivery of agents that make the tumor cells more susceptible to chemotherapy. Thus, the TfR can potentially open new avenues of combination therapies for the treatment of human cancers.

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