Effects of Transferrin Conjugates of Artemisinin and Artemisinin Dimer on Breast Cancer Cell Lines

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Abstract. *Transferrin (Tf) conjugates of monomeric artemisinin (ART) and artemisinin dimer were synthesized. The two conjugates, ART-Tf and dimer-Tf, retained the original protein structure, and formed stable aggregates in aqueous buffer. ART-Tf induced declines in proteins involved in apoptosis (survivin), cell cycling (cyclin D1), oncogenesis (c-myelocytomatosis oncogene product (c-MYC)), and dysregulated WNT signaling (beta-catenin) in both the human prostate (DU145) and breast (MCF7) cancer cell lines. Both ART-Tf and dimer-Tf induced down-regulation of survivin, c-MYC and mutated human epidermal growth factor receptor-2 (ERBB2 or HER2) in the BT474 breast cancer cell line. To our knowledge, this is the first demonstration that an ART derivative can cause a decline of ERBB2 in a human cancer cell line. Potential mechanisms for the observed effects are presented. Both transferrin conjugates strongly inhibited the growth of BT474 cells in the same concentration range that the conjugates caused declines in the levels of ERBB2, survivin, and c-MYC, while showing essentially no toxicity towards MCF10A normal breast cells.*

Artemisinin is a naturally-occurring peroxide, isolated from the Chinese medicinal plant, *Artemisia annua* L. Artemisinin and its simple derivatives have been widely used for malaria treatment in humans (1, 2). Clinical trials have demonstrated the remarkable anti-malarial activity of artemisinin derivatives and their excellent safety profiles (3, 4). Artemisinin derivatives, such as dihydroartemisinin and artesunate, also exhibit modest anticancer activity (5-7). Both anti-malarial and anticancer activities of artemisinin derivatives have been linked to iron-induced activation of the endoperoxide group of artemisinin which generates toxic radical species in the cells. Due to their rapid rate of division, most cancer cells have high rates of iron uptake (8). The enzyme ribonucleotide reductase, which converts ribose into deoxyribose, requires iron as a co-factor. Thus, during the S and G_1 phases of cell division when DNA replication occurs, transferrin receptors (TfR) are expressed on the cell surface to facilitate iron influx into the cell. Cancer cells, being in a condition of uncontrolled growth, have a higher concentration of cell surface TfR and take up higher amounts of iron from the circulation than normal cells do (8-12). We have shown that the anticancer activity of artemisinin is greatly enhanced by delivering the compound to the cellular iron uptake pathway (13, 14). Artemisinin-tagged transferrin (ART-Tf) has been demonstrated to have selective toxicity against Molt4 leukemia cells, with a selectivity of 34,000 when compared with normal lymphocytes (13). The selectivity is significantly higher than that of traditional anticancer agents such as Taxol. ART-Tf induces apoptosis through the cytochrome *c*-mediated intrinsic pathway (15). Additionally, we have shown that cancer cell toxicity of ART-Tf is dependent on the expression level of TfR in a prostate cancer cell line (15).

Recently, covalent dimers of artemisinin (ART dimers) have been shown to have significantly higher anti-malaria and anticancer activities compared to monomeric artemisinin derivatives (16-19). Some of the dimers are able to cure malaria in a mouse model in a single administration (20). We evaluated the cytotoxicity of one of the ART dimers, dimer-2Py, against a panel of four prostate cancer cell lines, DU 145, PC-3, C4-2, and LNCaP (21). While dimer-2Py was highly potent, dihydroartemisinin, a monomeric artemisinin, exhibited negligible activity under the same conditions. Dimer-2y reduced survivin protein levels in all of the prostate cancer cell lines. Interestingly, dimer-2Py also induced the loss of androgen receptor (AR) and prostate-

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Key Words: Artemisinin, transferrin, breast cancer, HER2, survivin, c-Myc, cyclin D1, DV145, MCF7 cells.

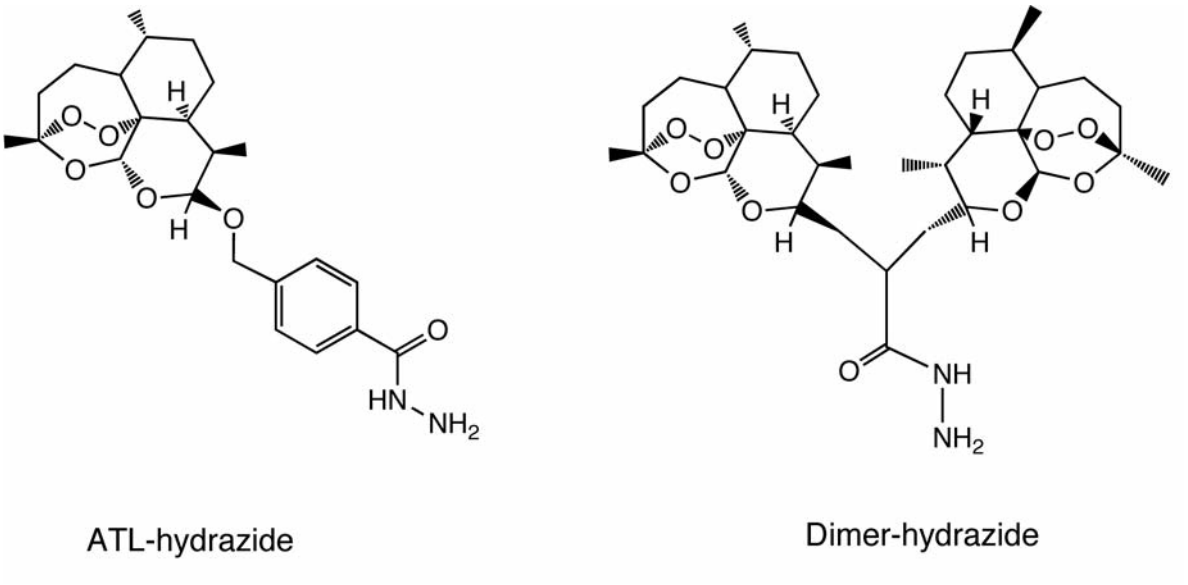

Figure 1. *Structures of artelinic acid hydrazide (ATL-hydrazide) and trioxane dimer hydrazide (dimer-hydrazide).*

specific antigen (PSA) expression in the C4-2 and LNCaP cells. The AR promotes both androgen-dependent and castration-resistant prostate tumor growth. Although at present it is not clear why covalent linking of artemisinin results in a significant increase in biological activities, ART dimers are an attractive class of compounds for further investigation. Recently, we were able to conjugate an ART dimer to transferrin by using the same conjugation protocol developed for ART-Tf. We wish to report here the synthesis of the two Tf conjugates, ART-Tf and dimer-Tf, and their effects on breast cancer cell lines *in vitro*.

Materials and Methods

BT474, MCF7, MCF10A and DU145 cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in Dulbecco's Modified Eagle Medium (DMEM) with L-glutamine (Invitrogen Corp., Grand Island, NY, USA) and supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA, USA) and penicillin/streptomycin). ART-Tf and dimer-Tf were dissolved in phosphate-buffered saline (PBS) and added to the cell culture medium.

The compounds dimer-hydrazide and artelinic acid hydrazide were synthesized according to published procedures (13, 14, 21, 22). Human holo-transferrin was obtained from Fortune Biologicals Inc. (Gaithersburg, MD, USA). Other chemicals and reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA), and used without purification. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-MS) data were obtained at the Medicinal Chemistry Mass Spectrometry Facility at the University of Washington. Circular dichroism spectra were recorded on Jasco 720 Spectropolarimeter (Jasco Corporation, Easton, MD, USA) at the University of Washington.

Preparation of dimer-Tf and ART-Tf. The procedure described below is a slight modification of the original literature procedure (13, 15). We found that the new procedure gave more consistent conjugation efficiency and higher *in vitro* activities. To a solution of 25 mg human holotransferrin, dissolved in 1 ml of 0.1 M sodium acetate buffer (pH 5.5), was added freshly-prepared sodium periodate solution (21.4 mg in 1 ml same buffer); the final concentration of sodium periodate was 50 mM. The resulting solution was incubated in the dark for 30 min at room temperature. The mixture was applied to gel filtration (Sephadex G-25, 1.5×25 cm) with 0.1 M sodium acetate buffer (pH 5.5) to remove excess periodate and other low molecular weight impurities. The protein fractions with an absorption value at 280 nm higher than 0.2 were combined, resulting in a solution of oxidized transferrin of around 7 ml. To the oxidized transferrin solution was added dimer hydrazide (Figure 1; 4 mg in 400 μl DMSO), which was then incubated in the dark for 20 h at ambient temperature. The reaction mixture was then dialyzed against deionized water to remove salt and excess trioxane dimer. The final product was lyophilized and stored at –20˚C. The dimer-Tf sample was further characterized by MALDI-MS to determine the average number of the trioxane dimer per Tf. ART-Tf was prepared in a similar way, using 4 mg of artelinic acid hydrazide (ATL-hydrazide, Figure 1) in 400 μl DMSO added to the oxidized transferrin solution to react for 2 h. The average number (±error) of dimer-hydrazide and ATL-hydrazide in their Tf conjugates were calculated to be 6.6(±0.1) and 6.3(±0.2), respectively.

Size-exclusion chromatography of holo-transferrin, ART-Tf and dimer-Tf. Solutions of native holo-transferrin, ART-Tf and dimer-Tf at 10 mg/ml were loaded separately on a size-exclusion column (Sephacryl S200, 1.5×75 cm), using 1 ml each and the column was eluted with Dulbecco's Phosphate-Buffered Saline (DPBS) buffer. Fractions were collected and measured for the absorbance at 280 nm. As a standard to calibrate the efficacy of the column, 2 mg of blue dextran were dissolved in 1 ml of DPBS buffer and passed through

the same column under the same conditions. Fractions were tested for absorbance at 620 nm. To test the optical properties of holo-transferrin and the conjugates, solutions of each sample at 6 μM in DPBS buffer were prepared.

Cell viability assay. The cytotoxicity of ART-Tf and dimer-Tf towards the BT474 breast cancer cell line and a control mammary epithelial cell line, MCF10A, was determined using the CytoTox 96 non-radioactive cytotoxicity kit from Promega (Madison, WI, USA), which measures the release of lactate dehydrogenase (LDH) from cells upon lysis. In brief, BT474, and MCF10A cells (5000 cells/100 μl) were seeded on a 96-well microplate and 100 μl culture medium containing ART-Tf or dimer-Tf were added to each well. After 72 h of incubation at 37˚C, the media were removed, and replaced with 150 μl of lysis buffer . After 45-min incubation at 37˚C, 50 μl of the supernatants were transferred to a new flat-bottom 96-well plate. Fifty microliters of reconstituted substrate mixture were added to each well. After incubating for 30 min at room temperature, 50 μl of stop solution were added and absorbance was measured at 490 nm on a Victor 3 plate reader (Perkin Elmer, Waltham MA, USA).

Western blot analysis. Whole-cell lysates were prepared as described previously (15). Protein levels were determined using the Bio-Rad D_C Protein Assay kit (BioRad Laboratories, Hercules, CA, USA). Western blotting was performed as described previously (15). Antibodies to β-catenin, DNA topoisomerase II alpha, mutated human epidermal growth factor receptor 2 (ERBB2) and cyclin D1 were from Epitomics (Burlingame, CA, USA). Antibodies to survivin and c-myelocytomatosis oncogene product (c-MYC) were from Cell Signaling Technology (Danvers, MA, USA), and to β-actin (clone AC-15) was from Sigma-Aldrich (St. Louis, MO, USA).

Results

Synthesis of Tf conjugates of artemisinin derivatives. Dimer-hydrazide was conjugated successfully to the oxidized Tf through the glycan chain. The average number of dimers per Tf was comparable to that for ART-Tf, as determined by MALDI-MS. Although ART dimers are quite insoluble in water, dimer-Tf remained water soluble at concentrations used in this study. The far-UV circular dichroism (CD) spectra of holo-transferrin, ART-Tf and dimer-Tf shared very similar shape and intensity, indicating that there was little change in the secondary structure between the native protein and the protein in the conjugates (Figure 2a). The results were consistent with our previous studies (15). Compared to the far-UV CD data, the differences between proteins were more significant in the near-UV CD spectra (Figure 2b). The spectral region (250-300 nm) was related to the overall environment around aromatic amino acids, such as phenylalanine, tyrosine and tryptophan. The near-UV CD spectra suggested that the introduction of hydrophobic artemisinin residues onto the protein surface may have altered the protein structure in a subtle way.

We found that both ART-Tf and dimer-Tf form stable aggregates in aqueous solution. In the size-exclusion chromatography on a Sephacryl S200 column, ART-Tf and dimer-Tf were both eluted at the void volume, whereas native transferrin eluted in later fractions, consistent with its monomeric state in solution (Figure 3). The aggregates were quite stable, and remained unchanged in the presence of 2 M guanidine-HCl, and 0.2% Tween 80 (data not shown).

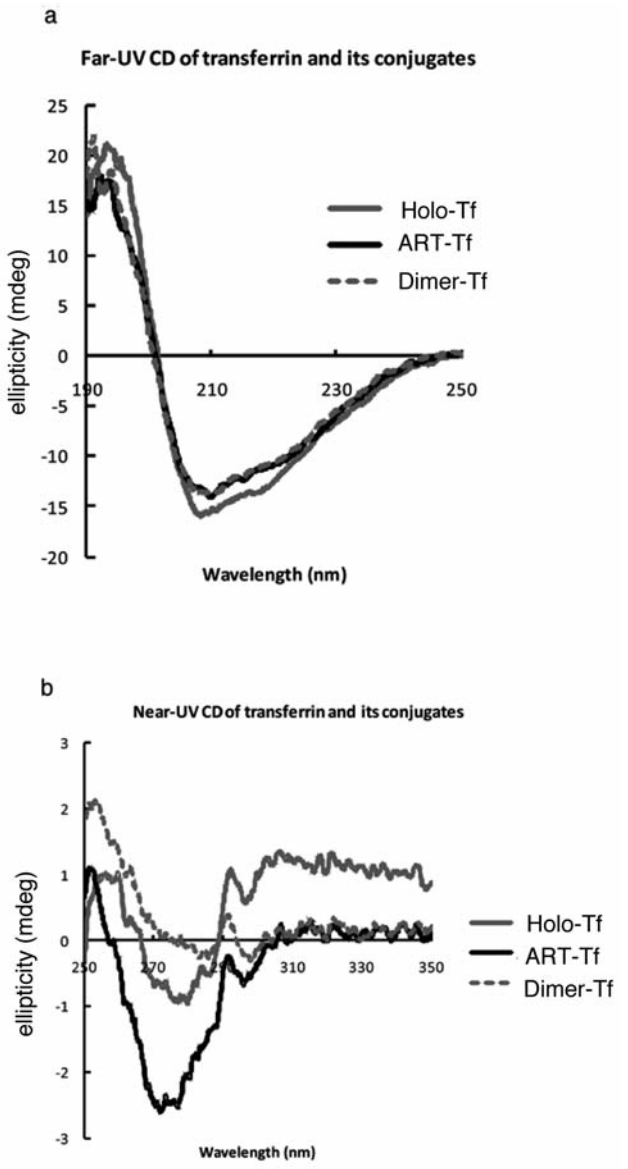

Figure 2. *The conjugation process has little effect on the secondary structure of the protein moiety. a: Far-UV circular dichroism (CD) spectra of holo-transferrin (grey line), ART-Tf (dark line) and dimer-Tf (dashed line) are similar to each other in shape. Protein concentrations were 2.8, 2.6 and 2.7 μM in 10 mM phosphate buffer, pH 7, for Tf, ART-Tf and dimer-Tf. b: Near-UV CD spectra of holo-transferrin (grey line), ART-Tf (dark line) and dimer-Tf (dashed line) showing substantial differences in the region of 250-300 nm. Protein concentrations were 28, 26 and 27 μM in 10 mM phosphate buffer, pH 7, for Tf, ART-Tf and dimer-Tf, respectively.*

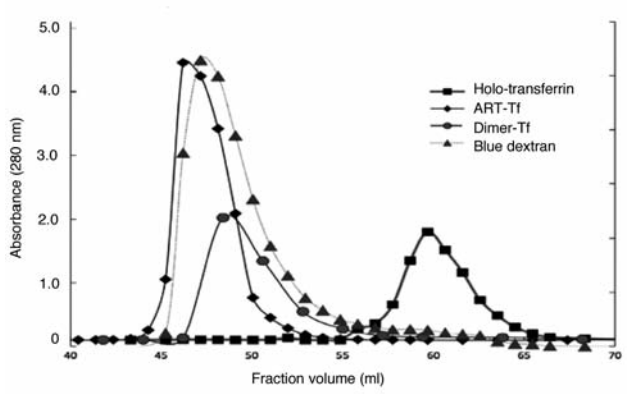

Figure 3. *Size exclusion chromatography of ART-Tf and dimer-Tf on Sephacryl S200 column. Both ART-Tf and dimer-Tf eluted in the same fractions as blue dextran (triangles), while holo-Tf eluted in later fractions that are consistent with its molecular weight (75 Kda).*

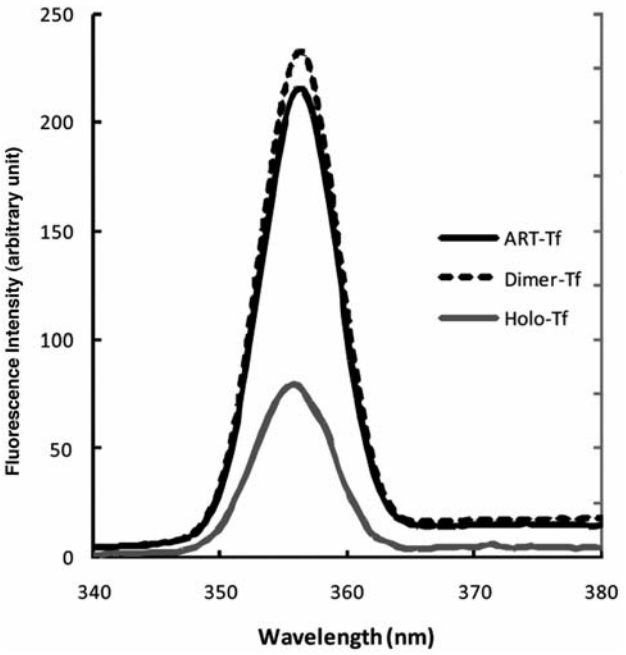

Figure 4. *The protein conjugates, ART-Tf and dimer-Tf, have stronger fluorescence intensities than holo-Tf at 356 nm, when excited at 280 nm. The concentration for each protein was 30 μM.*

Fluorescence spectra of proteins are sensitive to the change in the microenvironment around aromatic residues. When excited at 280 nm, ART-Tf, dimer-Tf and holo-transferrin had the same emission wavelength, but both artemisinin conjugates exhibited higher fluorescence intensities at the same concentration (Figure 4). The increase in fluorescence quantum yield may be due to protein aggregation, where the protein fluorophores would be shielded from aqueous environments.

Selected effects of ART-Tf on DU145 and MCF7 cell lines. We previously demonstrated that ART-Tf induces apoptosis in the DU145 cell line, causing mitochondrial cytochrome *c* release and activation of caspase-9 and -3 (15). In searching for changes in anti- and pro-apoptotic protein levels in several human prostate cancer cell lines, DU145, C4-2, and LNCaP, we found that an ART dimer suppressed levels of the anti-apoptotic protein survivin in all cell lines, but that levels of pro-apoptotic BCL2-associated X protein (BAX) and anti-apoptotic B-cell lymphoma-2 (BCL2) (23) did not change (21), nor did levels of the anti-apoptotic proteins, X-linked inhibitor of apoptosis protein (XIAP) and cellular inhibitor of apoptosis-1 (c-IAP-1) (unpublished data). Because dihydroartemisinin (DHA) reduced survivin mRNA and protein levels in a human cancer cell line (24), we determined whether levels of the transcription factor beta-catenin declined in DU-145 and MCF7 cell lines, since there is a beta-catenin/transcription factor (TCF) promoter upstream of the survivin gene (BIRC5) (25). Both survivin and beta-catenin levels were suppressed by ART-Tf in DU 145 and MCF7 cell lines (Figure 5). The *c-MYC* and *cyclin* D1 genes have a promoter for beta-catenin/TCF; the levels of these two proteins are frequently determined as 'read-outs' for the activity of the bipartite transcription factor beta-catenin/TCF (26, 27). The levels of both c-MYC and cyclin D1 declined in both cell lines after incubation with ART-Tf (Figure 5). Because beta-catenin/TCF forms a functional complex with DNA topoisomerase II alpha (28), we examined the levels of this enzyme in DU145 cells, and found that they declined (Figure 5); however, DNA topoisomerase II alpha levels were elevated (n=3 experiments) in MCF7 cells in the presence of ART-Tf (Figure 5). Thus, the levels of specific proteins involved in resistance to apoptosis (survivin), cell cycling (cyclin D1), oncogenesis (c-MYC) and dysregulated (29) WNT signaling (beta-catenin) decreased in both DU145 and MCF7 cell lines, while DNA topoisomerase II alpha levels declined in DU145 but increased in MCF7 cells.

Effects of ART-Tf and ART- dimer-Tf on survivin, ERBB2 and c-MYC levels in the BT474 cell line. The human breast cancer cell line BT474 overexpresses the mutated epidermal growth factor (EGF) receptor ERBB2 (HER2). Overexpression of ERBB2 in human breast tissue gives rise to 25% of all breast cancer cases (30). In BT474 cells, ERBB2 up-regulates survivin levels (31). Both ART-Tf and dimer-Tf caused strong declines in survivin levels at micromolar and sub-micromolar levels in BT474 cells (Figure 6a), as well as an approximate 50% decline in ERBB2 levels after 24 h of incubation.

Likewise, ART dimers caused strong declines in ERBB2 levels in the rat breast cancer cell line MTLn3 (data not shown). ART-Tf has been shown to retard the growth of MTLn3 tumors in rats (32).

Since c-MYC may regulate survivin levels in BT474 cells under certain conditions (33), and a c-MYC promoter is upstream of the survivin gene (*BIRC5*) (25), we determined whether c-MYC levels would be affected by 1 μM ART-Tf or 1 μM dimer-Tf. As in other cell lines treated with an artemisinin derivative (21) (Figure 5), both ART-Tf and dimer-Tf reduced c-MYC levels in BT474 cells (Figure 6b). Under the same conditions, there were no changes induced by either artemisinin conjugate in levels of beta-catenin (data not shown), whose promoter is also upstream of the survivin gene (25).

Both ART-Tf and dimer-Tf have selective toxicity towards breast cancer cells but not normal breast cells. Cancer cells, including breast cancer cells, are known to have up-regulated expression of TfR (8-12). Therefore, we hypothesized that the conjugation of Tf would result in increased selectivity for cancer cells. As shown in Figure 7, both ART-Tf and dimer-Tf effectively killed BT474 breast cancer cells at a concentration range that induced no toxicity in MCF10A cells. For BT474, the half-maximal inhibitory concentration (IC_{50}) of ART-Tf is 430 nM, and the one for dimer-Tf is 13 nM, indicating that dimer-Tf is approximately 30-fold more potent than ART-Tf. Notably, at the concentration of 0.1 μM, dimer-Tf caused almost 100% toxicity in BT474 cells, yet it had no effect at all on the viability of MCF10A cells.

The selectivity of ART-Tf for breast cancer cells is consistent with our previous finding that ART-Tf has selective toxicity against Molt4 leukemia cells when compared with normal lymphocytes (13). The current data suggest that dimer-Tf not only has selectivity, similarly to ART-Tf, but also has enhanced potency, with a lower IC_{50}.

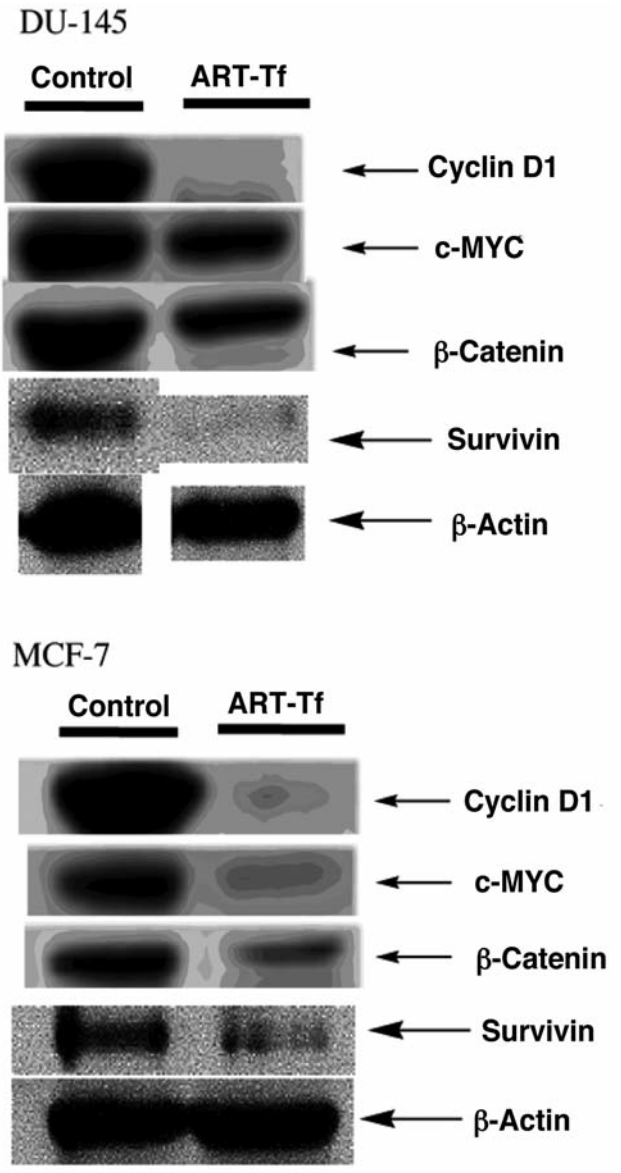

Figure 5. *ART-Tf induced changes in the levels of proteins involved in resistance to apoptosis, oncogenesis, cell cycling, and DNA synthesis in DU145 (prostate cancer) and MCF7 (breast cancer) cell lines. Cells were treated with 80 μM ART-Tf for 48 h and processed as described in the Materials and Methods section. ART-Tf concentration is given in artemisinin, with an average of 8 ART molecules/Tf. Results are representative of n=3 independent (consecutive) experiments.*

Discussion

The glycosidic chains of Tf provide an ideal place for artemisinin derivatives to conjugate to the protein surface because they are not involved in binding to TfR (34). Our data show that both ART-Tf and dimer-Tf form stable aggregates, while native Tf remains monomeric under the same experimental conditions. The aggregation is likely caused by the introduction of hydrophobic artemisinin moieties on the carbohydrate chains that are located in the C-terminus domain. Since the N-terminus domain remains hydrophilic, the Tf conjugates become amphiphilic, undergoing spontaneous association with each other through hydrophobic interaction. The aggregate formation is probably responsible, in part, for the increase in fluorescence quantum yield and subtle changes in near UV-CD of the Tf conjugates. Covalently linked Tf oligomers exhibited altered intracellular trafficking after endocytosis, resulting in longer intracellular retention and increased lysosomal degradation of Tf (35). When drugs such as doxorubicin (36) and methotrexane (35) are conjugated to oligomeric Tf, both drug delivery and cytotoxicity were increased compared to monomeric Tf drug conjugates. Although the aggregates of ART-Tf and dimer-Tf are formed

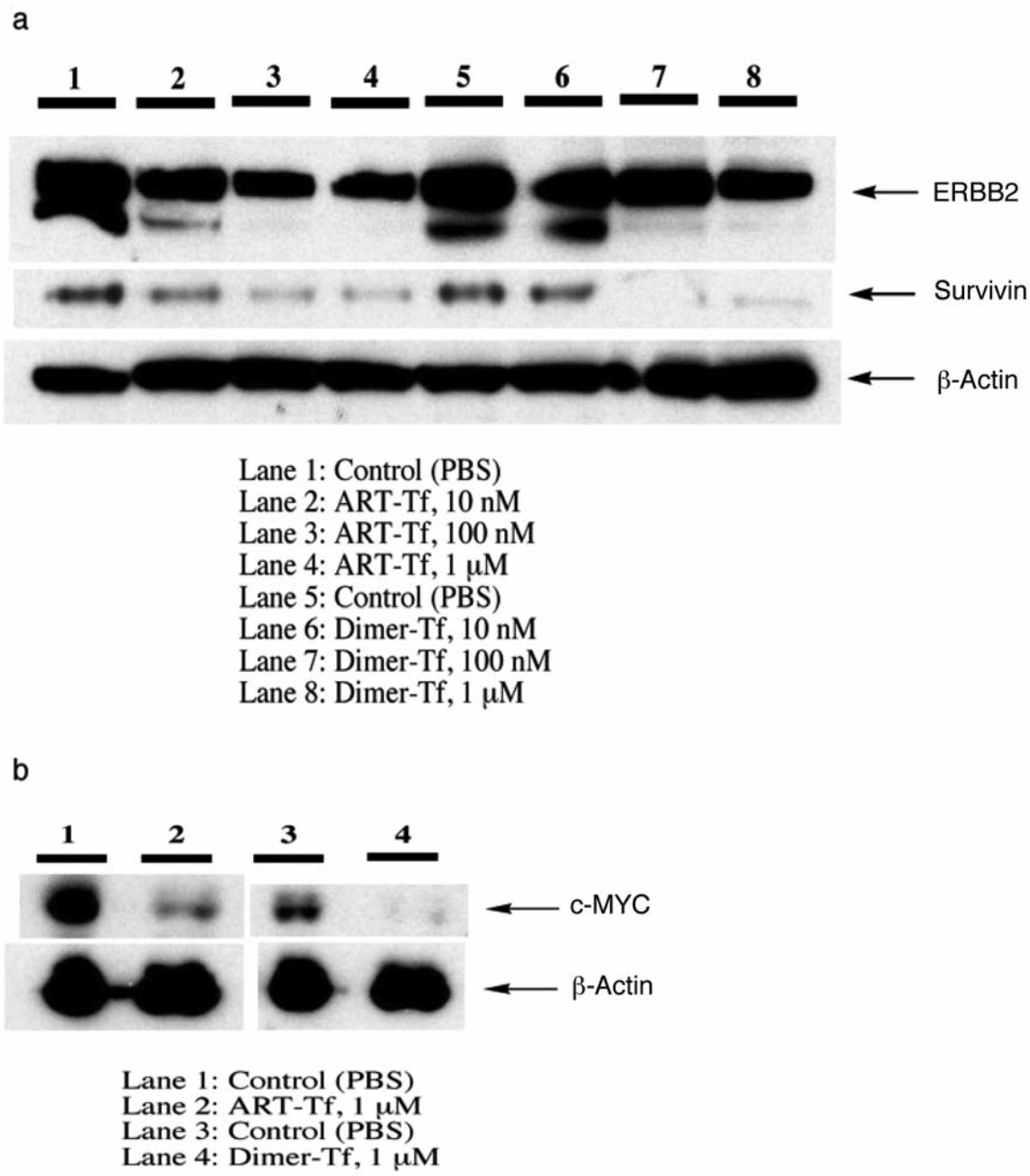

Figure 6. *a: Effects of ART-Tf and dimer-Tf on survivin and ERBB2 levels in BT474 breast cancer cells. BT474 cells were incubated for 24 h with diluent (PBS), 10 nM, 100 nM, or 1 μM ART-Tf (lanes 1-4) or diluent (PBS), 10 nM, 100 nM, or 1 μM dimer-Tf. b: Effects of ART-Tf and dimer-Tf on c-MYC levels in BT474 cells. BT474 cells were incubated for 24 h with diluent (lanes 1 and 3) 1 μM ART-Tf (lane 2) or 1 μM ART dimer-Tf (lane 4). The cells were harvested and processed as described in Materials and Methods. Representative of n=3 experiments. ART-Tf and dimer-Tf concentrations are given in artemisinin and artemisinin-dimer concentrations, respectively.*

non-covalently, they are quite stable and are likely to have similar advantages to monomeric Tf conjugates. The aggregate formation might also suppress the potential immunogenicity of the Tf conjugates by shielding the artemisinin-modified carbohydrate chain from the immune system.

Both ART-Tf and dimer-Tf induce a unique set of cellular responses in cancer cells. The variability in the responses of DU145 and MCF7 to ART-Tf treatment may represent the genetic variability observed in tumors. ART-Tf and dimer-Tf significantly down-regulated survivin, an anti-apoptotic protein

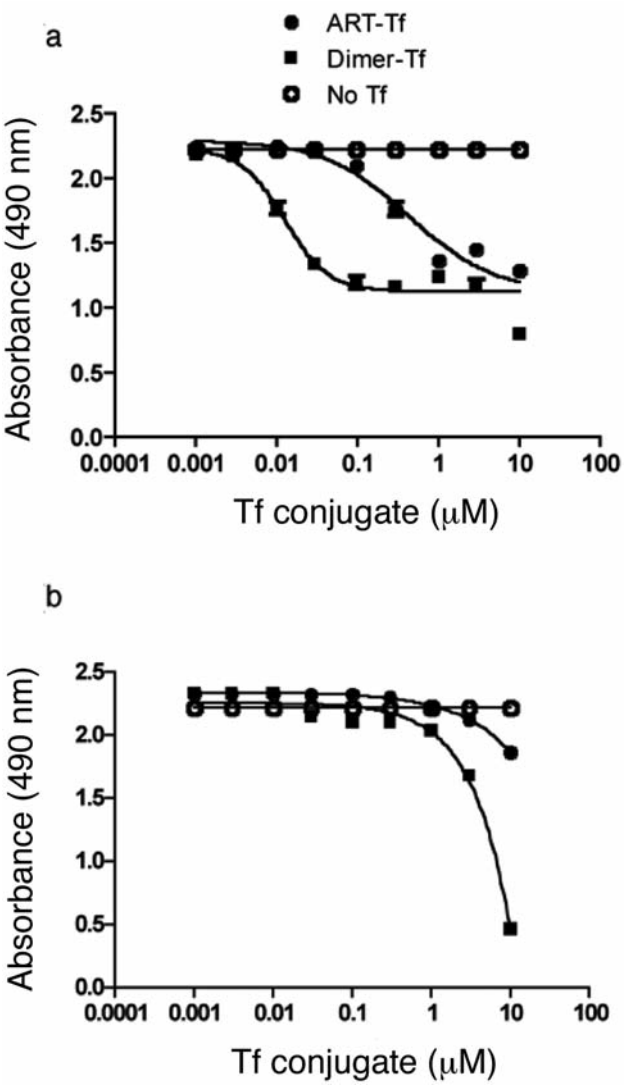

Figure 7. *ART-Tf and ART dimer-Tf have selective toxicity towards breast cancer cells but not to normal mammary epithelial cells. Results of viability assay are shown testing the dose-dependent toxicity of ART-Tf and dimer-Tf against breast cancer cell line BT474 (a) and a normal mammary epithelial cell line MCF10A (b). The cells were treated with 1:3 serial dilutions of ART-Tf or dimer-Tf in the range of 0.001-10 μM for 72 h. Cells in the control group were cultured in medium-alone. The IC_{50} for each compound was determined using the statistical software Graphpad Prism. The IC_{50}s for ART-Tf and dimer-Tf were 0.44 μM and 0.013 μM, respectively. The IC_{50}s for MCF10A could not be determined in the tested dose range.*

expressed in the majority of human cancers (37) and in human cancer cell lines, such as DU145, MCF7 and BT474 cells. *Survivin* mRNA levels were suppressed by dihydroartemisinin in a lung cancer cell line (24). Similarly, artesunate inhibited *survivin* mRNA expression in a colorectal cancer cell line (38), along with *c-MYC* mRNA and the beta-catenin protein. Since there are promoters for both *c-MYC* and *beta-catenin* upstream of the *survivin gene,* it is possible that ART derivative-induced declines in these two transcription factors cause declines in survivin levels. The results presented here, which show simultaneous suppression of beta-catenin and c-MYC, both potential up-regulators of *survivin* gene expression, also suggest that these two proteins may be involved in the regulation of survivin levels in prostate and breast cancer cell lines.

Survivin is undetectable in terminally differentiated adult tissues and is a key regulator of cell survival and cell-cycle progression. Suzuki *et al*. (39) have shown that survivin interacts with cyclin-dependent kinase(Cdk)-4, leading to Cdk2/cyclin-E activation and retinoblastoma protein (Rb) phosphorylation. As a result of survivin/Cdk4 complex formation, the CDK inhibitor p21 is released from its complex with Cdk4 and interact with mitochondrial procaspase-3 to suppress cell death (39). Therefore, the loss of survivin, cyclin-D1, and possibly CDK4, could considerably reduce proliferation and promote a pro-apoptotic phenotype in cancer cells. The remarkable tumor cell selectivity of ART-Tf and dimer-Tf may be due, at least in part, to the elevated iron metabolism of cancer cells and to the specific down-regulation of survivin. Survivin is a unique therapeutic target for cancer treatment although such an approach has not been widely explored. YM155, a small-molecule survivin suppressant, is currently undergoing clinical trials with promising results (40, 41).

ART-Tf also down-regulates cyclin D1 levels in DU145 and MCF7 cell lines. Among the cyclin isoforms, over-expression of cyclin D1 is most frequently associated with human cancer (42). Cyclin D1 is an important positive regulator of the G_1 to S-phase transition, which then commits the cell to DNA replication and cell division. Reduction of cyclin D1 levels by ART derivatives may be sufficient to arrest cancer cell growth (43).

ART-Tf also suppresses levels of c-MYC in the DU145, MCF7, and BT474 cell lines and dimer-Tf similarly reduces levels of c-MYC in the BT474 cell line. c-MYC is not only a transcription factor that drives the synthesis of many mRNAs (44), but it also enhances protein synthesis and is directly involved in promoting DNA replication (45).

ART-Tf reduced beta-catenin levels in DU145 and MCF7 cell lines. Artesunate also reduced beta-catenin levels in colorectal cancer cell lines (46). Mutations or aberrant expression of WNT signaling components may promote dysregulation of the WNT/beta-catenin pathway, giving rise to many kinds of cancer (47, 48). Cancer may arise from aberrant WNT signaling because beta-catenin/TCF transcriptionally activates genes whose expressed proteins regulate growth, cell signaling, and apoptosis (*e.g.* c-MYC, cyclin D1, and survivin).

Both ART-Tf and dimer-Tf caused a strong decrease in survivin levels and an approximate 50% decline in ERBB2

(HER2) receptor expression at the same concentrations in BT474 cells. We speculate that the decline in ERBB2 is due to a disruption of the chaperone system that controls ERBB2 stability (49). HER2-positive and ER-negative breast cancer represents a more aggressive type of breast cancer with relatively poor prognosis (30). Herceptin (trastuzumab) is the first HER2-targeted agent approved for clinical use in patients with breast cancer. Herceptin and other therapeutic antibodies are believed to exert their cytotoxic effects primarily through antibody-dependent cellular cytotoxicity (ADCC). In HER2-positive breast cancer, there is a significant correlation between HER2 and survivin expression (50). Down-regulation of survivin could enhance cell killing efficiency when combined with trastuzumab-induced ADCC. ART-Tf down-regulates c-MYC, the primary nuclear target of HER2-HER3 heterodimer. Inhibiting c-MYC results in accumulation of p27, a kinase inhibitor controlling cell-cycle progression, which induces cell-cycle arrest at the G_1 phase. c-MYC is over-expressed in about 30% of patients with HER2-positive breast cancer (51).

Artemisinin and its diverse derivatives induce apoptosis in many different human cancer cell lines (52). Likewise, different derivatives of artemisinin, such as an ART dimer (21) and ART-Tf, cause similar changes, that is, declines in the levels of the same proteins in different human cancer cell lines. ART dimer-2Py caused declines in the levels of survivin, cyclin D1, and c-MYC in DU145, C4-2, and LNCaP prostate cancer cell lines (21), while ART-Tf caused declines in survivin, cyclin D1, c-MYC, and beta-catenin in DU145, as well as the MCF7 breast cancer cell line (Figure 5). Survivin levels are also reduced in the SPCA1 lung cancer cell line by DHA (24) and in colorectal cancer cell lines (46). Artemisinin (52) and the ART-dimer (21) each cause a loss of the AR in C4-2 and LNCaP cell lines. Very likely due to their different genetic backgrounds and to the difference in signaling pathways in each cell line, the same changes are not observed in all cell lines, such that beta-catenin does not decrease in C4-2 and LNCaP cells, nor does DNA topoisomerase II alpha decrease in MCF7, while declining in DU145 cells. However, the failure of a cell line to exhibit a decline in the level of a particular protein in response to an ART derivative, when other cell lines have exhibited such a decline, is the exception. Further mechanistic details would require the identification of initial cellular target(s) of ART. In summary, various ART derivatives cause declines in most of the same dysregulated proteins involved in aberrant WNT signaling (beta-catenin) and in resistance to apoptosis (survivin), cell cycling (cyclin D1), and oncogenesis (c-MYC) in different cancer cell lines. These diverse changes suggest that ART derivatives may be useful in treating different types of human cancer.

Both ART-Tf and dimer-Tf showed remarkable selectivity towards tumor cells when they were tested on BT474 and MCF10A cells. The high selectivity is consistent with our previous work on leukemia cells and normal lymphocytes. Cancer cells are generally more sensitive to artemisinin compounds compared to normal cells due to their elevated iron metabolism. Survivin also plays a crucial role in maintaining the proliferation of cancer cells, while it is undetectable in normal cells. Specific down-regulation of survivin by ART-Tf or dimer-Tf may be responsible for the high selectivity observed.

In conclusion, Tf conjugates of artemisinin derivatives can maintain these hydrophobic drugs in solution and may deliver them to tumor sites selectively because of high expression of the TfR on the surface of cancer cells. The ability of these artemisinin conjugates to down-regulate numerous proteins involved in the genesis and maintenance of cancer cells suggests that these compounds may be beneficial in treating different neoplasms. Further pre-clinical development of the Tf conjugates of artemisinin are underway in our laboratory.

Conflicts of Interest

University of Washington owns patents on artemisinin-transferrin conjugates in which TS and HL are listed as co-inventors. Holleypharma is a licensee of the technology for commercial development.

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