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Effects of artemisinin-tagged holotransferrin on cancer cells

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Abstract

Artemisinin reacts with iron to form free radicals that kill cells. Since cancer cells uptake relatively large amount of iron than normal cells, they are more susceptible to the toxic effect of artemisinin. In previous research, we have shown that artemisinin is more toxic to cancer cells than to normal cells. In the present research, we covalently attached artemisinin to the iron-carrying plasma glycoprotein transferrin. Transferrin is transported into cells via receptor-mediated endocytosis and cancer cells express significantly more transferrin receptors on their cell surface and endocytose more transferrin than normal cells. Thus, we hypothesize that by tagging artemisinin to transferrin, both iron and artemisinin would be transported into cancer cells in one package. Once inside a cell, iron is released and can readily react with artemisinin close by tagged to the transferrin. This would enhance the toxicity and selectivity of artemisinin towards cancer cells. In this paper, we describe a method to synthesize such a compound in which transferrin was conjugated with an analog of artemisinin artelinic acid via the N-glycoside chains on the C-domain. The resulting conjugate ('tagged-compound') was characterized by MALDI-MS, UV/Vis spectroscopy, chemiluminescence, and HPLC. We then tested the compound on a human leukemia cell line (Molt-4) and normal human lymphocytes. We found that holotransferrin-tagged artemisinin, when compared with artemisinin, was very potent and selective in killing cancer cells. Thus, this 'tagged-compound' could potentially be developed into an effective chemotherapeutic agent for cancer treatment.

Keywords: Artemisinin; Transferrin tagging; Cancer cells

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Introduction

An important aspect of cancer chemotherapy is to design drugs that have high potency and specificity in killing cancer cells. In this paper, we describe the synthesis of a compound that has these properties. This involves the covalent tagging of artemisinin analogs to the N-glycoside moiety of holotransferrin.

Artemisinin is a sesquiterpene lactone isolated from the plant Artemisia annua L. The compound and its analogs are being used as an antimalarial and their pharmacology and pharamcokinetics have been well studied (Dhingra et al., 2000; Li and Wu, 2003; Navaratnam et al., 2000). Artemisinin contains an endoperoxide that could react with an iron atom to form a carbon-based free radical. Such free radical, when formed intracellularly, could cause macromolecular damages and lead to cell death. Since cancer cells uptake a large amount of iron compared to normal cells, they are more vulnerable to the cytotoxic effect of artemisinin than normal cells. Our previous research (Lai and Singh, 1995; Singh and Lai, 2001) have shown that, in vitro, Molt-4 cells, a human leukemia cell line, and human breast cancer cells are more susceptible to the cytotoxic effect of artemisinin than their normal counterparts (i.e., human lymphocytes and normal breast cells, respectively). The LD_{50} for Molt-4 cells is approximately 100 times less than that of lymphocytes. Further research has shown that artemisinin induces mainly apoptosis in cancer cells (Singh and Lai, 2004). Various other researchers have also reported the potential anticancer properties of artemisinin and its analogs (Beekman et al., 1997, 1998; Chen et al., 2003, 2004; Efferth et al., 2001; 2002; Efferth and Oesch, 2004; Jeyadevan et al., 2004; Lee et al., 2000; Li et al., 2001; Mukanganyama et al., 2002; Posner et al., 1999, 2003, 2004; Reungpatthanapong and Mankhetkorn, 2002; Sadava et al., 2002; Sun et al., 1992; Woerdenbag et al., 1993, Wu et al., 2001).

In mammalian cells, iron is transported into the cytoplasm via a receptor-mediated endocytosis process (Andrews, 2000). Binding of the plasma iron-carrying protein transferrin to cell surface transferrin receptors triggers endocytosis. A drop in pH in the endosome causes the release of iron from transferrin. Iron is then actively pumped out into the cytoplasm. Transferrin and transferrin receptors are recycled back to the cell surface. Since cancer cells require a large amount of iron, e.g., as a cofactor in the synthesis of deoxyriboses before cell division, they express a high number of transferrin receptors on their surface. For example, breast cancer cells have 5–15 times more transferrin receptors on their cell surface than normal breast cells (Reizenstein, 1991), and transferrin receptors are expressed on cell surface of breast carcinoma cells but not on benign breast tumor cells (Raaf et al., 1993). Breast cancer cells do take up more iron than normal breast cells (Shterman et al., 1991).

We speculate that if artemisinin is covalently attached to holotransferrin (iron-loaded transferrin), it would be transported in the same package into cells and react with the iron within the endosome where iron would be released from holotransferrin. This may enhance the cytotoxic potency and selectivity of artemisinin on cancer cells.

Transferrin is a glycoprotein. Its protein moiety is mainly involved in its binding to cell surface transferrin receptors, whereas the carbohydrate chains are not involved in receptor binding (Mason et al., 1993). Transferrin has two N-glycosides attached to Asn residues in the C-terminal domain (Van Halbeek et al., 1981). Periodate oxidation of these carbohydrate chains generate reactive aldehyde groups that can be modified with a variety of hydrazine or aminoxy derivatives of artemisinin. Assuming that all 1,2-diol moieties are oxidized to the corresponding aldehyde group, we estimate

that as many as 10 artemisinin derivatives could be tagged to a molecule of transferrin. Thus, we have tagged an artemisinin analog artelinic acid to the gycosylate-moiety of holotransferrin using a relatively simple process. Holotransferrin was first treated with $NaIO_4$ to oxidize the N-glycoside chains to expose aldehyde groups on the surface. Artelinic acid hydrazide was then reacted with the oxidized holotransferrin to form a covalent conjugate (the 'tagged-compound'). Mass spectral analysis showed that the 'tagged-compound' contained on an average of 4 artelinic acid moieties per molecule.

In this paper, we report a method to synthesize this 'tagged-compound' and the results of testing the compound on Molt-4 cells (a human leukemia cell line) and normal human lymphocytes. We compared the potency of the 'tagged-compound' with dihydroartemisinin, an artemisinin analog.

In addition, we tested the potency of a compound in which artelinic acid was attached to lysine residues in holotransferrin. To prepare this compound, holotransferrin was reacted with artelinic acid and N-ethyl-N′ -dimethylaminopropylcarbodiimide (EDC). Thus, reactive lysine residues on the protein surface could be acylated by artelinic acid. It is expected that this latter compound would be less potent than the 'tagged-compound' because attachment of artelinate to lysines would interfere with the binding of holotarnsferrin to transferrin receptors.

Material and Methods

Synthesis of artemisinin-tagged compounds

General

All starting materials and reagents for organic synthesis were purchased from Sigma-Aldrich (St. Louis, MO) and used without further purification. Dihydroartemisinin was a gift from Holley Pharmaceuticals (Fullerton, CA). All reactions were carried out in oven-dried glassware under an inert atmosphere of nitrogen. Flash column chromatography was carried out with EM type 60 (230–400 mesh) silica gel. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker 500 MHz DRX Avance FT-NMR spectrometer at frequencies 499.85 and 202.34 MHz, respectively. MALDI-MS was recorded on Bruker Biflex III Matrix Assisted Laser Desorption Ionization Time of Flight Mass spectrometer. Low-resolution mass spectra were recorded on Bruker Esquire Liquid Chromatograph-Ion Trap mass spectrometer. pH was measured with a Radiometer Copenhagen PHM84 pH meter. UV/Vis spectra were acquired on Perkin-Elmer Lambda 3B Spectrophotometer using 2-cm, 1-cm or 1-mm cells. HPLC was performed using a Waters 600E system equipped with a Perkin Elmer LC-95 UV-Vis detector.

Synthesis of methyl 4-[(10-dihydroartemisininoxy) methyl] benzoate of artemisinin

Dihydroartemisinin was first acetylated with acetic anhydride in pyridine in the presence of 4-(dimethylamino)pyridine as described by Kim and Sasaki (2004). To a solution of the acetylated artemisinin (compound 1 in Fig. 1) (0.100 gm, 0.306 mmol) and methyl p-hydroxymethylbenzoate (0.056 gm, 0.336 mmol) in anhydrous $CHCl_3$ (1 ml) was added TMSOTf (0.013 ml, 0.06 mmol) with stirring at room temperature. After 40 min, the reaction mixture was quenched with saturated $NaHCO_3$ solution (0.5 ml) and extracted with $CHCl_3$. The organic layer was washed with water and brine, dried over Na_2SO_4 and concentrated under vacuo. The residue obtained was purified by flash column

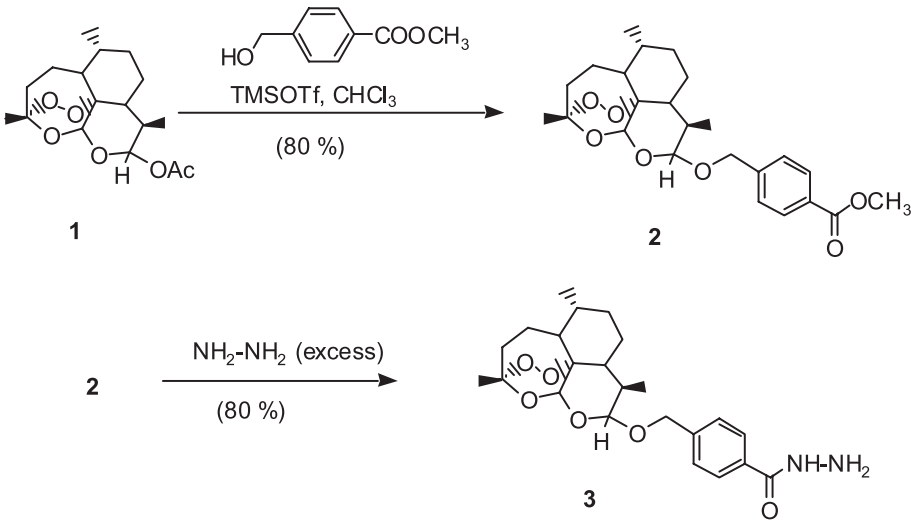

Fig. 1. Synthesis of artelinic acid hydrazide (3) from dihydroartemisinin (1).

chromatography using 20% EtOAc / Hexane to give artelinic acid methyl ester (compound 2 in Fig. 1) (0.105 gm, 80%). The product was identical to that reported in the literature (Shrimali et al., 1998).

Synthesis of 4-[(10-dihydroartemisininoxy) methyl] benzoic acid hydrazide (3)

To a solution of the ester compound (compound 2 in Fig. 1) (0.100 gm, 0.231 mmol) in ethanol (0.2 ml) was added hydrazine hydrate (0.046 ml) and the reaction mixture was heated at 50°C for 48 hrs. The reaction mixture was concentrated under vacuo and water (2 ml) was added to the reaction mixture and extracted with $CHCl_3$. The combined organic extracts were dried over Na_2SO_4, concentrated under vacuo and the crude product obtained was purified by flash column chromatography using 4% MeOH / $CHCl_3$ to give the final artelinic acid hydrazide (Compound 3 in Fig. 1) (0.080 gm, 80%). ^{1}H NMR ($CDCl_3$) δ 0.97 (m, 7H), 1.28 (m, 2H), 1.47 (brs, 4H), 1.64 (m, 1H),1.83 (m, 2H), 1.89 (m, 1H), 2.07 (m, 1H), 2.39 (dt, 1H), 2.71(brs, 1H), 4.57 (d, J= 12.9 Hz, 1H), 4.94 (m, 2H), 5.46 (s, 1H), 7.39 (d, J= 9.9 Hz, 2H), 7.75 (d, J= 9.9 Hz, 2H); ^{13}C NMR ($CDCl_3$) δ 13.50, 20.72, 24.92, 25.06, 26.56, 31.29, 34.97, 36.79, 37.82, 44.73, 52.93, 69.51, 81.48, 88.44, 101.98, 104.60, 127.44, 127.65, 143.04; LRMS (EI): *m/z* 433 $(M+H)^+$.

Oxidation and Conjugation of oxidized transferrin with artelinic hydrazide (Fig. 2)

Human holotransferrin (1 gm) dissolved in 50 ml, 0.1 M sodium acetate pH 5.5 was incubated at room temperature with 15 ml, 50 mM sodium periodate ($NaIO_4$) for 30 min. The reaction mixture was applied to a Sephadex G-25 column equipped with a UV detector in cold room and eluted with 0.1 M sodium acetate pH 5.5. The protein concentration of the oxidized sample (60 ml, 1.90 x10^{-4} M) was determined using the ε-values for transferrin ε_{280} = 92,300 M^{-1} cm^{-1}. Holotransferrin was oxidized with 10 mM $NaIO_4$ or 50 mM $NaIO_4$ before the tagging reaction with artelinic acid hydrazine. The tagging reaction was incomplete when 10 mM $NaIO_4$ was used.

A solution of artelinic hydrazide (compound 3 in Fig. 1) (0.120 gm) dissolved in DMSO (7.8 ml) was added to the oxidized transferrin (60 ml) and the reaction mixture was incubated at room temperature for 2 hrs. Low molecular weight reagents and byproducts were removed from the reaction mixture by gel

filtration on a Sephadex G-25 column equipped with a UV monitor in cold room, using DPBS saline buffer as an eluent. Protein containing fractions were pooled up to give a solution of artelinic tagged-transferrin (the 'tagged-compound') (2.77×10^{-4} M).

Preparation of lysine-tagged holotransferrin

Human holotransferrin (0.050 gm) dissolved in 2.5 ml 0.1 M sodium acetate (pH 5.5) was incubated at room temperature for 30 min with 0.750 ml, 50 mM $NaIO_4$. The reaction mixture was applied to a Sephadex G-25 column in a cold room and eluted with 0.1 M HEPES buffer at pH 8.0. The elution profile was monitored by UV absorbance at 280 nm. Protein fractions were pooled up to give the final oxidized transferrin (1.17×10^{-4} M).

1-Hydroxybenzotriazole (6 mg) was added to a solution of artelinic acid (6 mg) in DMSO (0.012 ml) followed by the addition of EDC (6 mg). The reaction mixture was incubated at room temperature for 1.5 hr, diluted with 0.378 ml DMSO and added to 3 ml of the above oxidized transferrin solution in HEPES buffer. The reaction mixture was incubated at room temperature for a further 2 hrs, centrifuged and purified by gel filtration on a Sephadex G-25 column in a cold room, using DPBS saline buffer as an eluent. Protein containing fractions were pooled up to give the lysine-tagged transferrin (1.90×10^{-4} M).

Chromatographic separation of the tagged transferrins (Hydrophobic Interaction Chromatography)

Hydrophobic Interaction Chromatography equipped with an UV detector (280 nm) was used to analyze the tagged proteins (the 'tagged-compound' and lysine-tagged transferrin). A 40 μl volume of native and tagged proteins was applied to a Polypropyll A HPLC column (PolyLC, 1000 Å pore size) and the column was eluted with a linear salt gradient from 2 M ammonium sulfate (pH 6.5) to 0.1 M potassium phosphate (pH 6.5) at a flow rate of 1 ml/min.

Chemiluminescence Measurements

Dihydroartemisinin (0.020 gm) was weighed in a 100 ml glass volumetric flask and then dissolved in MeOH. The flask was filled to the mark with methanol to give a final stock concentration of 200 μg/ml. A chemiluminescence reagent consisting of a mixture of a solution of luminol (15 μg/ml) and hematin (30 μg/ml) in 0.1 M NaOH was prepared and was allowed to stand for 30 min before use. Dihydroartemisinin stock solution (10 μl) was added to 1 ml of this chemiluminescence reagent and the chemiluminescence was measured at 25°C after setting the emission wavelength and slit width at 425 nm and 20 nm, respectively. Similarly, the chemiluminescence of the tagged-compound was measured and a graph of intensity vs time was plotted.

Effect of synthetic compounds on human cells

Molt-4-lymphoblastoid cells and human lymphocytes were used in this study. Molt-4 cells were purchased from the American Typed Culture Collection (Rockville, MD). They are acute lymphoblastic leukemia cells from human peripheral blood. Cultures were maintained in RPMI-1640 (Gibco, Long Island, NY) supplemented with 10% fetal bovine serum (Hyclone, New Haven, CT). Cells were cultured at 37°C in 5% CO_2/95% air and 100% humidity, and were split 1:2 at a concentration of approximately 1×10^6/ml. Cell concentration before an experiment was between 150×10^3–300×10^3 per ml. Human lymphocytes were isolated from fresh blood obtained from a healthy donor using a

modification of the Ficoll-hypaque centrifugation method of Boyum (1968). In this method, 20–100 μl of whole blood obtained from a finger prick were mixed with 0.5 ml of ice-cold RPMI-1640 without phenol red (GIBCO, NY) in a 1.5-ml heparinized microfuge tube (Kew Scientific Inc., Columbia, OH). Using a Pipetman, 100 μl of cold lymphocyte separation medium (LSM) were layered at the bottom of the tube. The samples were centrifuged at 3500 rev/min for 2 min in a microfuge (Sorvall, Microspin model 245) at room temperature. Lymphocytes in the upper portion of the Ficoll layer were pipetted out. Cells were washed twice in 0.5 ml of RPMI-1640 by centrifugation for 2 min at 3500 rev/min in a microfuge. The final pellet, consisting of approximately 0.4–2.0 $\times$ 10^5 lymphocytes, was resuspended in RPMI-1640. Cell viability was determined before experiments using trypan blue exclusion and found to be > 95%.

Cells (Molt-4 and lymphocytes) were aliquoted in 0.1 ml volumes into microfuge tubes. Different concentrations (3.1, 6.2, 12.4 μM) of a test compound were added to the tubes. Control samples were added the same volume of the medium. For the effects of dihydroartemisinin and artelinic acid, human holotransferrin (12 μM) was first added to some cell samples. Different concentrations (3.1, 6.2, 12.4 μM) of freshly prepared dihydroartemisinin or artelinic acid dissolved in complete medium were added 1 hr later to the tubes. Cells were kept in an incubator at 37°C under 5% CO_2 and 95% air during the experiment. At 24, 48, and 72 hrs after the addition of the compounds, cell number was counted from a 10-μl aliquot from the samples using a hemocytometer. Cells were thoroughly mixed by repeated pipeting before an aliquote was taken for counting. In the case of Molt-4 cells, cell viability was not determined because it is not correlated with cell loss.

Data are expressed as percentage of cell count at a certain time-point compared to cell count at the time when a test compound was added (time zero in figures). Time-response curves were compared by the method of Krauth (1980). The levels of the curves, i.e., a_o of the orthogonal polynomial coefficient, were compared with the median test. χ^2 was calculated with Yate's correction for continuity. A difference of $p < 0.05$ was considered statistically significant. The Probit analysis was used to determine LD_{50}s, i.e., the concentration of the test compound that causes a decrease in cell count by 50% in 72 hrs, from the dose-response data.

Results

Synthesis of artelinic acid hydrazide and tagged transferrin

Overall steps of synthesis of artelinic acid hydrazide is shown in Fig. 1. The first coupling step gave a reasonably high yield of the artelinate ester by using a new TMSOTf-mediated reaction (Kim and Sasaki, 2004). In the second step, the endoperoxide bond in artemisinin did not react with hydrazine, a strong reducing agent, even with an elevated temperature and a prolonged reaction time. The overall yield of artelinic acid hydrazide is 64%, starting from dihydroartemisinin.

The tagging reaction is shown in Fig. 2. We found that oxidized transferrin undergoes slow decomposition even when stored at 4°C. Thus, the oxidized transferrin was rapidly purified by gel-filtration, and reacted immediately with artelinic acid hydrazide dissolved in DMSO. The tagging reaction proceeded smoothly, and no precipitate was formed during the reaction. The 'tagged-compound', when stored at 4 °C, was found to be stable with no lost in cytotoxic activity for at least 6 months.

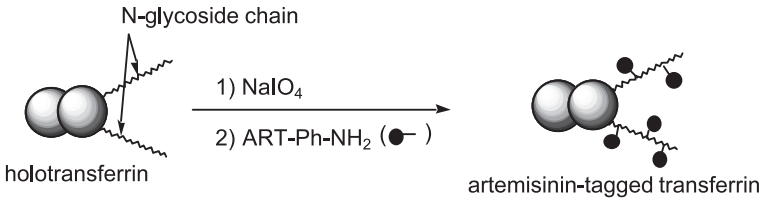

Fig. 2. Synthesis of artelinate-tagged transferrin. The two circles represent N-and C-terminal domains of transferrin. Both N-glycoside chains are attached to Asn residues in the C-terminal domain. They are oxidized with NaIO4, and then reacted to artelinic acid hydrazide.

Spectroscopic characterization of the tagged transferrin

UV/Vis spectroscopy was initially used to characterize the tagged transferrin. The absorbance ratio (A_{470nm}/A_{280nm}) for holotransferrin and tagged-transferrin were 0.042 and 0.017 respectively. Thus, the iron content of the tagged-transferrin was only 40% compared to that of native holotransferrin. $NaIO_4$ is known to cause oxidative damages to tyrosine residues and hence the tyrosine residue at the iron-binding site of transferrin may have been partially oxidized after the tagging reaction. We attempted to minimize the loss of iron by limiting the oxidizing reagent, shortening the reaction time, or increasing the reaction pH. However, these modified reaction conditions also resulted in the formation of incompletely tagged transferrins.

Chromatographic characterization of tagged transferrin

We were able to separate the tagged proteins and native transferrin by hydrophobic interaction HPLC (Fig. 3). Since artemisinin is a hydrophobic compound, the tagged proteins are expected to

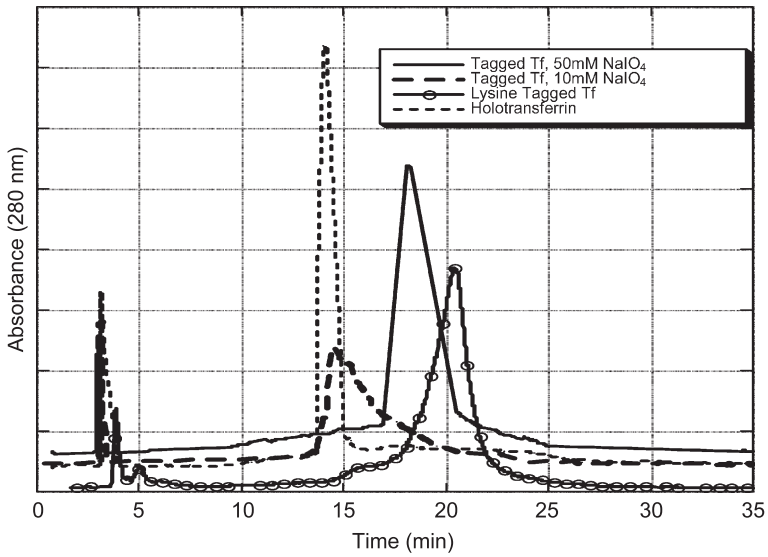

Fig. 3. Hydrophobic interaction chromatographic separation of holotransferrin and tagged-transferrins POLYPROPYLL A column (200 × 4.6 mm). Mobile phase: A = 2.0 M $(NH_4)_2SO_4$(pH 6.5); B = 0.1 M KH_2PO_4 (pH 6.5). Gradient conditions: 0% B to 100% B in 15 min, hold at 100% B for 25 min, 100% B to 0% B in 5 min; flow rate 1 ml/min; temperature 21 °C; wavelength 280 nm; 0.5 AUFS.

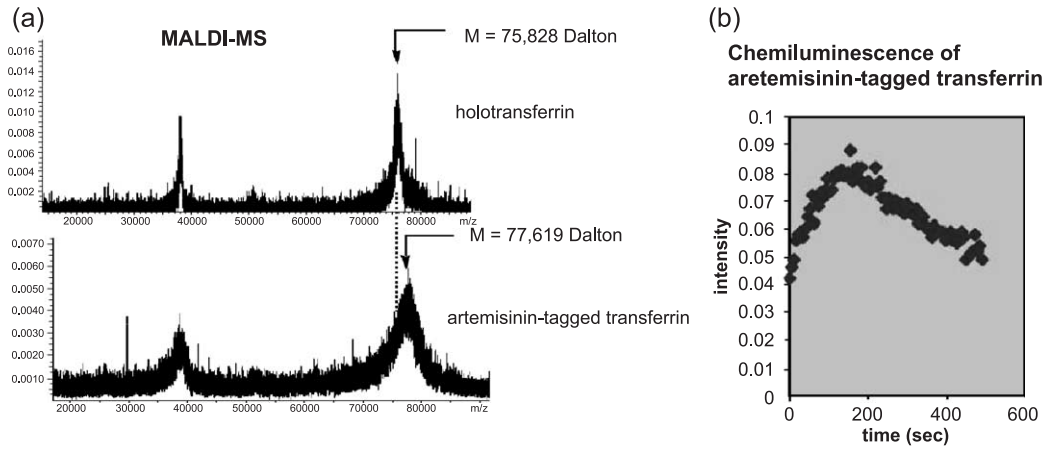

Fig. 4. (a) MALDI-MS analysis of human transferrin and the 'tagged-compound'. (b) Chemiluminescence analysis of the 'tagged-compound'.

move slower on HPLC as compared to the native transferrin. The retention times for the 'tagged-compound' and native transferrin were 18 min and 14 min, respectively. The peak of the 'tagged-compound' was much broader than that of native transferrin, suggesting that our sample was a mixture of tagged proteins with different numbers of artelinic acid molecules on the protein surface. The lysine-tagged transferrin had a comparable retention time with that of the carbohydrate-based tagged transferrin, indicating that the number of artelinic acid moieties per protein is similar in these tagged transferrins. MALDI-MS further confirmed the HPLC data. The 'tagged-compound' and native transferrin gave their molecular ion peaks at 77,619 Dalton and 75,828 Dalton, respectively (Fig. 4a). The mass difference (1,791 Dalton) corresponds to the tagging of 4.1 artemisinin moieties (molecular weight = 432) per protein on average. The peak shape of the 'tagged-compound' was much broader than that of native transferrin, consistent with the HPLC data.

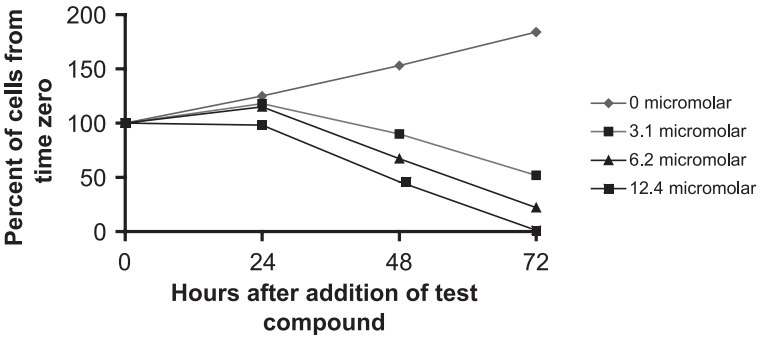

Fig. 5. Effects of the 'tagged-compound' on Molt-4 cells. Different concentrations of the compound were added to cell cultures at time zero and cells were counted at different times later. Data are expressed as cell count as percentage from that at time zero. Each curve represents data from three experiments.

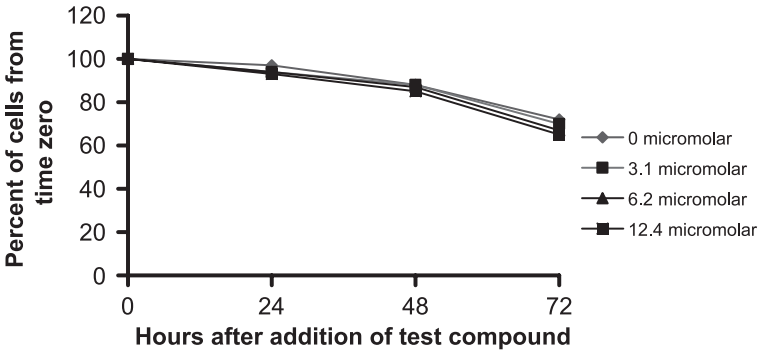

Fig. 6. Effects of the 'tagged-compound' on human lymphocytes. Different concentrations of the compound were added to cell cultures at time zero and cells were counted at different times later. Data are expressed as cell count as percentage from that at time zero. Each curve represents data from three experiments. There is no significant difference among the response curves.

The endoperoxide bond is intact in tagged transferrin

We used chemiluminescence to show that the endoperoxide bond was intact in the artemisinin moieties of the tagged protein. Both dihydroartemisinin and artemisinin produce chemiluminescence when mixed with hematin and luminol due to their endoperoxide bond (Green et al., 1995). When the 'tagged-compound' was reacted with the chemiluminescence reagent (hematin and luminol), the solution produced a time dependent chemiluminescence similar to that of dihydroartemisinin (Fig. 4b). No attempt was made to determine the number of endoperoxide groups per protein based on chemiluminescence because the reaction was very sensitive to the structure of artemisinin and surrounding environment.

Effects of the synthetic compounds on human cells

The 'tagged-compound' exerts a dose-dependent cytotoxicity on Molt-4 cells (Fig. 5). Each curve is significantly different from another (χ^2 = 4.5, df =1, $p < .035$). It is effective at relatively low concentration (3.1 μM) and acts slowly. On the other hand, the 'tagged-compound' is relatively ineffective in killing normal lymphocytes. No significant difference was detected among the different doses tested (Fig. 6).

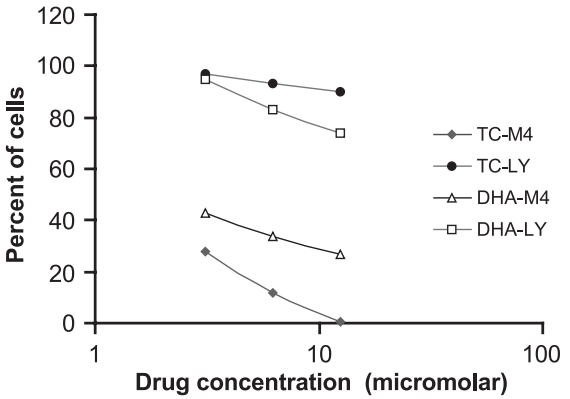

Fig. 7. Log dose-response curves of Molt-4 (M4) and lymphocytes (LY) to the 'tagged-compound' (TC) and dihydroartemisinin (DHA). Each curve includes data from three experiments.

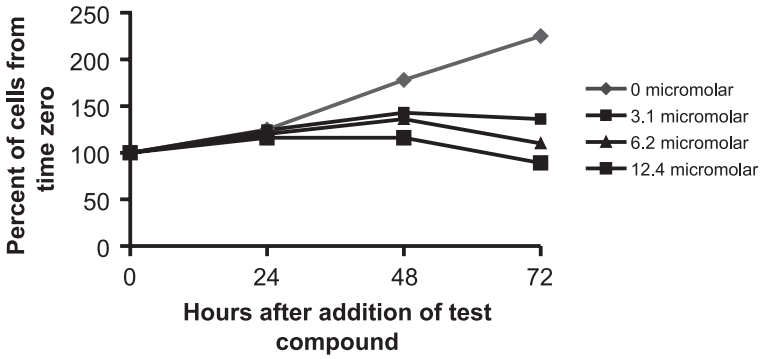

Fig. 8. Effects of artelinic acid on Molt-4 cells. Holotransferrin (12 μM) was added to cell cultures at 1 hr before time zero. Different concentrations of artelinic acid were then added to the cultures at time zero and cells were counted at different times later. Data are expressed as cell count as percentage from that at time zero. Each curve represents data from four experiments.

Fig. 7 plots the dose-response at 72 hrs after treatment with the 'tagged-compound' or dihydroartemisinin on Molt-4 cells and human lymphocytes. The following are the LD_{50}s of the 'tagged-compound' and dihydrartemisinin (DHA) on Molt-4 and lymphocytes as determined by the Probit analysis: Molt-4-'tagged-compound' 0.98 μM; Molt-4-DHA 1.64 μM; lymphocyte-'tagged compound' 33 mM; lymphocyte-DHA 58.4 μM. Thus, compared to DHA, the 'tagged-compound' is more potent in killing Molt-4 cells and less potent in killing normal lymphocytes. Dihydroartemisinin is 36 times more potent in killing Molt-4 cells than its normal counterpart, whereas for the tagged-compound, it is 34000 times. Therefore, tagging artemisinin to holotransferrin has greatly increased the specificity of the cancer cell killing property of artemisinin.

Effects of artelinate on Molt-4 cells are presented in Fig. 8. It is significantly less potent in killing the cancer cells compared to the 'tagged-compound'. Also, data presented in Fig. 9 show that 'holotransferrin with artelinate tagged to lysine residues' is also less effective in killing Molt-4 cells than the 'tagged-

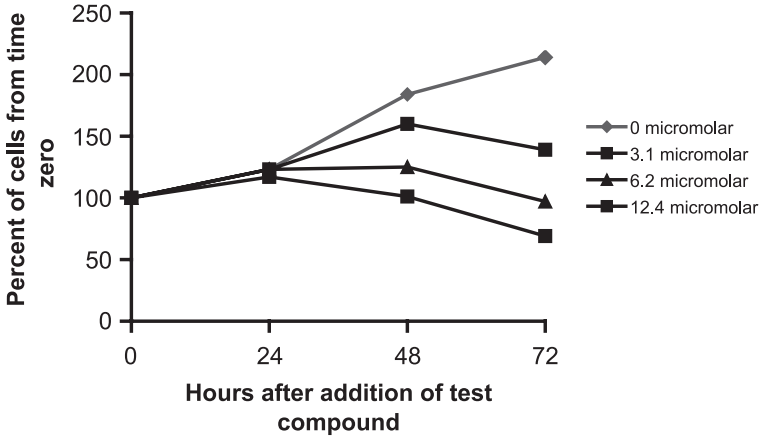

Fig. 9. Effects of holotransferrin with artelinate attached to lysine residues on Molt-4 cells. Different concentrations of the compound were added to cell cultures at time zero and cells were counted at different times later. Data are expressed as cell count as percentage from that at time zero. Each curve represents data from three experiments.

compound'. However, it did decrease the number of cells in the culture, suggesting that some molecules might still be able to bind to receptors and were transported into cells.

Discussion

Artemisinin and its derivatives react rapidly with iron when they are mixed in solution. We were initially concerned about the stability of the' tagged-compound' because both iron and artemisinin are held closely in the compound. The UV/Vis and chemiluminescence data show that the tagged transferrin contains both iron and active artemisnin moieties. The partial loss of iron in the 'tagged-compound' occurs during the oxidation step. After the tagging reaction, the 'tagged-compound' is very stable when stored at 4°C at neutral pH. The remarkable stability of the 'tagged-compound' could be attributed to the low iron dissociation constant of transferrin under neutral pH.

The cytotoxicity data indicate that the 'tagged-compound' is a very potent and selective killer of Molt-4 cells. It is also more potent than dihydroartemisinin. For artemisnin derivatives to kill cells, they must be delivered into a cell where iron or other redox active metal ions can activate the endoperoxide group to generate radical species. While DHA enters into cells by simple diffusion, the 'tagged-compound' requires receptor-mediated endocytosis. The fact that tagging to lysine residues signifcantly reduced the potency indicates that endocytosis of the 'tagged-compound' plays a role in the enhanced potency. In addition, artelinic acid itself shows only a weak cytotoxic effect. Artelinic acid is deprotonated in a neutral condition, making it too hydrophilic to cross the cell membrane.

Use of artemisinin for cancer treatment is limited by its fast elimination from the plasma (Alin et al., 1996; Dhingra et al., 2000; Navaratnam et al., 2000). Peak concentration after an oral adminstration is very short lasting. Since transferrin is a natural component in the blood, it is expected that the 'tagged-compound' could last longer in the circulation for it to react with cancer cells. Furthermore, cancer cell development of resistance to the 'tagged-compound' is less likely. The 'tagged-compound' can be administered intravenously or in an aerosol inhalant (Li et al., 2003).

Another advantage of the 'tagged-compound' is that it provides a better chance for the tagged artemisinin to react with iron in the endosome to form harmful free radicals. Since cancer cells have more transferrin receptors than normal cells, the 'tagged-compound' thus concentrates artemisinin in cancer cells, whereas artemisinin, taken alone, would enter both cancer and normal cells equally. However, such enhancement in selectivity is not expected to be drastic in in vitro cell culture experiments. This is shown in the results of this experiment that the 'tagged-compound' is only approximately two times more potent than dihydroartemisinin in killing cancer cells. The observed enhanced selectivity of the 'tagged-compound' is thus due to the fact that the 'tagged-compound' is much less toxic to normal cells compared to DHA.

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References

Alin, M.H., Ashton, M., Kihamia, C.M., Mtey, G.J.B., Bjorkman, A., 1996. Clinical efficacy and pharmacokinetics of artemisinin monotherapy and in combination with mefloquine in patients with falciparum malaria. British Journal of Clinical Pharmacology 41, 587–592.

Andrews, N.C., 2000. Iron homeostasis: insights from genetics and animal models. Nature Reviews: Genetics 1, 208–217.

Beekman, A.C., Wierenga, P.K., Woerdenbag, H.J., Uden, W.V., Pras, N., Konings, A.W.T., El-Feraly, F.S., Galal, A.M., Wikstrom, H.V., 1998. Artemisinin-derived sesquiterpene lactones as potential antitumour compounds: cytotoxic action against bone marrow and tumour cells. Planta Medica 64, 615–619.

Beekman, A.C., Woerdenbag, H.J., Van Uden, W., Pras, N., Konings, A.W.T., Wikstrom, H.V., 1997. Stability of artemisinin in aqueous environments: impact on its cytotoxic action to Ehrlich ascites tumour cells. Journal of Pharmacy and Pharmacology 49, 1254–1258.

Boyum, A., 1968. Isolation of mononuclear cells and granulocytes from human blood. Scandinavian Clinical Laboratory Investigation 21, 77–89.

Chen, H.H., Zhou, H.J., Fang, X., 2003. Inhibition of human cancer cell line growth and human umbilical vein endothelial cell angiogenesis by artemisinin derivatives in vitro. Pharmacological Research 48, 231–236.

Chen, H.H., Zhou, H.J., Wu, G.D., Lou, X.E., 2004. Inhibitory effects of artesunate on angiogenesis and on expressions of vascular endothelial growth factor and VEGF receptor KDR/flk-1. Pharmacology 71, 1–9.

Dhingra, V., Rao, K.V., Narasu, M.L., 2000. Current status of artemisinin and its derivatives as antimalarial drugs. Life Sciences 66, 279–300.

Efferth, T., Dunstan, H., Sauerbrey, A., Miyachi, H., Chitambar, C.R., 2001. The anti-malarial artesunate is also active against cancer. International Journal of Oncology 18, 767–773.

Efferth, T., Davey, M., Olbrich, A., Rucker, G., Gebhart, E., Davey, R., 2002. Activity of drugs from traditional Chinese medicine toward sensitive and MDR1-or MDR1-overexpressing multidrug-resistant human CCRF-CEM leukemia cells. Blood Cells, Molecules, and Diseases 28, 160–168.

Efferth, T., Oesch, F., 2004. Oxidative stress response of tumor cells: microarray-based comparison between artemisinins and anthracyclines. Biochemical Pharmacology 68, 3–10.

Green, M.D., Mount, D.L., Todd, G.D., Capomacchia, A.C., 1995. Chemiluminescent detection of artemisinin novel endoperoxide analysis using luminol without hydrogen peroxide. Journal of Chromatography A 695, 237–242.

Jeyadevan, J.P., Bray, P.G., Chadwick, J., Mercer, A.E., Byrne, A., Ward, S.A., Park, B.K., Williams, D.P., Cosstick, R., Davies, J., Higson, A.P., Irving, E., Posner, G.H., O'Neill, P.M., 2004. Antimalarial and antitumor evaluation of novel C-10 non-acetal dimers of 10beta-(2-hydroxyethyl)deoxoartemisinin. Journal of Medicinal Chemistry 47, 1290–1298.

Kim, B.J., Sasaki, T., 2004. Synthesis of *O*-aminodihydroartemisinin via TMS triflate catalyzed C-O coupling reaction. Journal of Organic Chemistry 69, 3242–3244.

Krauth, J., 1980. Nonparametric analysis of response curves. Journal of Neuroscience Method 2, 239–252.

Lai, H., Singh, N.P., 1995. Selective cancer cell cytotoxicity from exposure to dihydroartemisinin and holotransferrin. Cancer Letters 91, 41–46.

Lee, C.H., Hong, H., Shin, J., Jung, M., Shin, I., Yoon, J., Lee, W., 2000. NMR studies on novel antitumor drug candidates, deoxoartemisinin and carboxypropyldeoxoartemisinin. Biochemical and Biophysical Research Communication 274, 359–369.

Li, Y., Wu, Y.L., 2003. An over four millennium story behind qinghaosu (artemisinin-a fantastic antimalarial drug from a traditional chinese herb). Current Medicinal Chemistry 10, 2197–2230.

Li, X., Fu, G.F., Fan, Y.R., Shi, C.F., Liu, X.J., Xu, G.X., Wang, J.J., 2003. Potent inhibition of angiogenesis and liver tumor growth by administration of an aerosol containing a transferrin-liposome endostatin complex. World Journal of Gastroenterology 9, 262–266.

Li, Y., Shan, F., Wu, J.M., Wu, G.S., Ding, J., Xiao, D., Yang, W.Y., Atassi, G., Leonce, S., Caignard, D.H., Renard, P., 2001. Novel antitumor artemisinin derivatives targeting G1 phase of the cell cycle. Bioorganic and Medicinal Chemistry Letter 11, 5–8.

Mason, A.B., Miller, M.K., Funk, W.D., Banfield, D.K., Savage, K.J., Oliver, R.W.A., Green, B.N., MacGillivray, R.T.A., Woodworth, R.C., 1993. Expression of glycosylated and nonglycosylated human transferrin in mammalian cells.

Characterization of the recombinant proteins with comparison to three commercially available transferrins. Biochemistry 32, 5472–5479.

Mukanganyama, S., Widersten, M., Naik, Y.S., Mannervik, B., Hasler, J.A., 2002. Inhibition of glutathione S-transferases by antimalarial drugs possible implications for circumventing anticancer drug resistance. International Journal of Cancer 97, 700–705.

Navaratnam, V., Mansor, S.M., Sit, N.W., Grace, J., Li, Q., Olliaro, P., 2000. Pharmacokinetics of artemisinin-type compounds. Clinical Pharmacokinetics 39, 255–270.

Posner, G.H., Ploypradith, P., Parker, M.H., O'Dowd, H., Woo, S.H., Northrop, J., Krasavin, M., Dolan, P., Kensler, T.W., Xie, S., Shapiro, T.A., 1999. Antimalarial, antiproliferative, and antitumor activities of artemisinin-derived, chemically robust, trioxane dimers. Journal of Medicinal Chemistry 42, 4275–4280.

Posner, G.H., Park, I.H., Sur, S., McRiner, A.J., Borstnik, K., Xie, S., Shapiro, T.A., 2003. Orally active, antimalarial, anticancer, artemisinin-derived trioxane dimers with high stability and efficacy. Journal of Medicinal Chemistry 46, 1060–1065.

Posner, G.H., McRiner, A.J., Paik, I.H., Sur, S., Borstnik, K., Xie, S., Shapiro, T.A., Alagbala, A., Foster, B., 2004. Anticancer and antimalarial efficacy and safety of artemisinin-derived trioxane dimers in rodents. Journal of Medicinal Chemistry 47, 1299–1301.

Raaf, H.N., Jacobsen, D.W., Savon, S., Green, R., 1993. Serum transferrin receptor level is not altered in invasive adenocarcinoma of the breast. American Journal of Clinical Pathology 99, 232–237.

Reizenstein, P., 1991. Iron, free radicals and cancer. Medical Oncology and Tumor Pharmacotherapy 8, 229–233.

Reungpatthanapong, P., Mankhetkorn, S., 2002. Modulation of multidrug resistance by artemisinin, artesunate and dihydroartemisinin in K562/adr and GLC/adr resistant cell lines. Biological and Pharmaceutical Bulletin 25, 1555–1561.

Sadava, D., Phillips, T., Lin, C., Kane, S.E., 2002. Transferrin overcomes drug resistance to artemisinin in human small-cell lung carcinoma cells. Cancer Letters 179, 151–156.

Shrimali, M., Bhattacharya, A.K., Jain, D.C., Bhakuni, R.S., Sharma, R.P., 1998. Sodium artelinate: a potential antimalarial. Indian Journal of Chemistry 37B, 1161–1163.

Shterman, N., Kupfer, B., Moroz, C., 1991. Comparison of transferrin receptors, iron content and isoferritin profile in normal and malignant human breast cell lines. Pathobiology 59, 19–25.

Singh, N.P., Lai, H., 2001. Selective toxicity of dehydroartemisinin and holotransferrin on human breast cancer cells. Life Sciences 70, 49–56.

Singh, N.P., Lai, H., 2004. Artemisinin induces apoptosis in human cancer cells. Anticancer Research 24, 2277–2280.

Sun, W.C., Han, J.X., Yang, W.Y., Deng, D.A., Yue, X.F., 1992. [Antitumor activities of 4 derivatives of artemisic acid and artemisinin B in vitro]. Zhongguo Yao Li Xue Bao 13, 541–543.

Van Halbeek, H., Dorland, L., Vliegenthart, J.F.G., Spik, G., Cheron, A., Montreuil, J., 1981. Structure determination of two oligomannoside-type glycopeptides obtained from bovine lactotransferrin, by 500 MHz proton NMR spectroscopy. Biochimica et Biophysica Acta 675, 293–296.

Woerdenbag, H.J., Moskal, T.A., Pras, N., Malingre, T.M., 1993. Cytotoxicity of artemisinin-related endoperoxides to Ehrlich ascites tumor cells. Journal of Natural Products 56, 849–856.

Wu, J.M., Shan, F., Wu, G.S., Li, Y., Ding, J., Xiao, D., Han, J.X., Atassi, G., Leonce, S., Caignard, D.H., Renard, P., 2001. Synthesis and cytotoxicity of artemisinin derivatives containing cyanoarylmethyl group. European Journal of Medicinal Chemistry 36, 469–479.