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Mini review

Anticancer properties of artemisinin derivatives and their targeted delivery by transferrin conjugation

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Abstract

Artemisinin and its derivatives are well known antimalaria drugs and particularly useful for the treatment of infection of Plasmodium falciparum malaria parasites resistant to traditional antimalarials. Artemisinin has an endoperoxide bridge that is activated by intraparasitic heme-iron to form free radicals, which kill malaria parasites by alkylating biomolecules. In recent years, there are many reports of anticancer activities of artemisinins both in vitro and in vivo. Artemisinins have inhibitory effects on cancer cell growth, including many drug- and radiation-resistant cancer cell lines. The cytotoxic effect of artemisinin is specific to cancer cells because most cancer cells express a high concentration of transferrin receptors on cell surface and have higher iron ion influx than normal cells via transferrin mechanism. In addition, some artemisinin analogs have been shown to have antiangiogenesis activity. Artemisinin tagged to transferrin via carbohydrate chain has also been shown to have high potency and specificity against cancer cells. The conjugation enables targeted delivery of artemisinin into cancer cells. In this review, we discuss the anticancer activities and mechanisms of action of artemisinins and the transferrin-conjugate.

Keywords: Artemisinin; Transferrin; Conjugation; Anticancer property; Cytotoxicity

1. Introduction

Artemisinin is a sesquiterpene lactone isolated from the plant *Artemesia annua* L. and is being widely used as an antimalaria drug. It is particularly effective against *P. falciparum* malaria parasites that are resistant to traditional antimalarial drugs (Dhingra et al., 2000; Meshnick, 2002; O'Neill, 2004; White, 2004).

Various derivatives of artemisinin, such as dihydroartemisinin, artemether, artesunate, and arteether have also potent activities against malaria parasites (Fig. 1) (Dhingra et al., 2000; Wiesner et al., 2003). The artemisinin derivatives act much faster than the other antimalarial drugs, with an approximate parasite- and fever-clearance time of 32 h versus 2–3 days (Wiesner et al., 2003). These compounds have a potent effect on chloroquine-resistant malarial parasites, and have been increasingly used for over 20 years (Wiesner et al., 2003). Despite the reported neurotoxic and embryotoxic effects in animals occurring at higher doses, application of artemisinins in humans seems to be relatively safe (Dhingra et al., 2000; Meshnick, 2002; Wiesner et al., 2003).

In recent years, artemisinin derivatives also have been shown to be effective in killing cancer cells (Lai and Singh, 1995; Efferth et al., 2001, 2004; Singh and Lai, 2001; Wu et al., 2004). Artemisinin kills cancer cells mainly by inducing apoptosis. Interestingly, artemisinin is essentially non-toxic to normal cells. In this mini-review, we discuss the current status of the anticancer activities and the mechanisms of action of artemisinins. We also discuss a unique drug delivery strategy that involves the attachment of cytotoxic drugs including artemisinin to transferrin via its surface carbohydrate chains.

2. Generation of free radicals by the interaction of artemisinin with iron

The artemisinin molecule contains an endoperoxide bridge (–C–O–O–C–) that interacts with a Fe(II) to form free radicals

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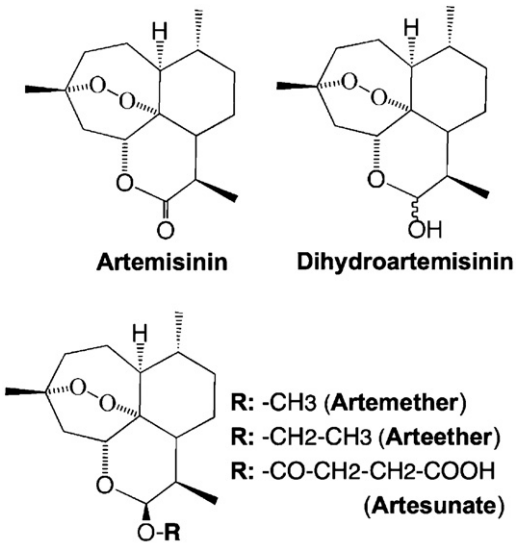

Fig. 1. Chemical structures of artemisinin and its derivatives.

(Rosenthal and Meshnick, 1996; Dhingra et al., 2000). An intact endoperoxide is crucial, since artemisinin derivatives lacking an endoperoxide bridge are devoid of antimalarial activity (Dhingra et al., 2000). Unlike Fe(II), Fe(III) does not cause reductive session of the endoperoxide. The reaction between artemisinin and Fe(III) is very slow, and the reaction products have been attributed to acid mediated heterolytic cleavage of the peroxide (Creek et al., 2005). Because malaria parasites contain a high amount of Fe(II) in the form of heme molecules (Wiesner et al., 2003), artemisinin's antimalarial bioactivity is due to its reaction with the intraparasitic iron source and generation of free radicals leading to cellular destruction (Meshnick et al., 1989; Dhingra et al., 2000).

Artemisinins are initially activated by the cleavage of the endoperoxide with intraparasitic heme-iron (Wu et al., 1998; Wiesner et al., 2003). Subsequent biochemical events and cellular target(s) of artemisinin, however, remain unclear. It has been proposed that the transfer of an oxygen atom from the peroxide group of artemisinins to the chelated iron generates a Fe(IV) = O species (Posner et al., 1995; Wu et al., 1998). The resulting free radical intermediate may kill the parasite by alkylating and damaging essential malarial proteins (Wu et al., 1998; Dhingra et al., 2000; Efferth et al., 2004). The antimalarial activities of artemisinins in vitro are enhanced by high oxygen tension and by addition of other free radical generating compounds such as doxorubicin, miconazole, and castecin (Dhingra et al., 2000).

3. Transferrin and expression of transferrin receptors on cancer cells

Iron plays a vital role in cell growth. Since the solubility of Fe(III) is very low at neutral pH, many living organisms have evolved sophisticated iron-transport and uptake mechanisms. Mammalian cellular iron acquisition mechanism involves the serum iron binding protein transferrin (cf. reviewed by Li and Qian, 2002; Qian et al., 2002; Baker et al., 2003; Cheng et al., 2004; Gomme et al., 2005). Transferrin is a medium size protein of approximately 700 amino acid residues. The protein has two domains (C-domain and N-domain) where iron binds to a cavity in each domain (Gomme et al., 2005). The binding affinity is very high ($K_d \sim 10^{-20}$ M) (Baker et al., 2003). Due to the high iron-binding affinity of transferrin, there is essentially no free iron in serum. This is believed to be a body's defense mechanism against pathogenic organisms. Iron-bound transferrin (holo-transferrin) binds to cell surface transferrin receptors with a high binding affinity ($K_d \sim 10^{-9}$ M) (Lee et al., 2001). The complex formation between transferrin and its receptor initiates a process called endocytosis where a depression on the membrane surface is initially formed and then separates into a vesicle (endosome). The pH inside the endosome drops to approximately pH 5.5, and the acidification induces a conformational change of transferrin that results in the release of bound Fe(III) (Li and Qian, 2002). The released Fe(III) is immediately reduced to Fe(II) by a membrane-bound reductase, and transported to the cytoplasm by a divalent metal transporter protein (DMT-1). The endosome is then recycled back to the membrane surface, and apo-transferrin (iron-free transferrin) is released from the receptors (Qian et al., 2002; Cheng et al., 2004). Each serum transferrin molecule undergoes 100–200 cycles of ion binding, intracellular transport, and release during its life time (Hamilton et al., 2004). The level of transferrin receptor expression varies depending on cell types. Non-dividing cells have relatively low levels of transferrin receptor expression, whereas rapidly proliferating cells can express up to 100,000 transferrin receptors per cell (Qian et al., 2002; Gomme et al., 2005). Most cancer cells express a high concentration of transferrin receptors on cell surface and have a high amount of Fe(III) ion uptake into cells (Qian et al., 2002; Gomme et al., 2005). For example, in the case of human breast cancer cells, 5–15 times more transferrin receptors are expressed on their cell surface compared to normal breast cells, and breast cancer cells take up more iron than normal breast cells (Reizenstein, 1991). High cell surface concentrations of transferrin receptor are also found in leukemic cells (Castañeda et al., 1991; Das Gupta et al., 1996; Efferth et al., 2004). The CCRF-CEM leukemia and U373 astrocytoma cells express transferrin receptor in 95% and 48% of the cell population, respectively, whereas only 0.4–1.3% of peripheral mononuclear healthy blood cells were express transferrin receptor (Efferth et al., 2004). Because cancer cells have higher iron influx rates (and presumably higher intracellular free iron) compared to the corresponding normal cells, cancer cells are more susceptible to the cytotoxic effect of artemisinins.

4. In vitro anticancer activity of artemisinin and its analogs

Dihydroartemisinin, an analogue of artemisinin, selectively killed Molt-4 lymphoblastoid cells when co-incubated with holo-transferrin, whereas the same treatment was significantly less toxic to normal human lymphocytes (Lai and Singh,

1995). The drug combination of dihydroartemisinin and holo-transferrin was approximately 100 times more effective on Molt-4 cells than normal lymphocytes (LC_{50}s of Molt-4 and normal lymphocytes were 2.6 μM and 230 μM, respectively) (Lai and Singh, 1995). Incubation with dihydroartemisinin alone was found to be less effective than in combination with holo-transferrin, indicating that intracellular iron plays a role in the cytotoxic effect.

HTB 27 cells, a radiation-resistant human breast cancer cell line, were killed effectively (reduced to 2% of original concentration) after 16 hr of treatment with dihydroartemisinin (200 μM) and holo-transferrin (12 μM) (Singh and Lai, 2001). However HTB 125 cells, a normal breast cell line, were not significantly affected by the same treatment. Also, when the breast cancer cells were treated with only dihydroartemisinin (200 μM) (without holo-transferrin), the cytotoxicity was significantly lower (Singh and Lai, 2001).

Artemisinin was tested on drug-sensitive H69 human small-cell lung carcinoma (SCLC) and multi-drug-resistant (H69VP) SCLC cells pretreated with holo-transferrin (Sadava et al., 2002). The cytotoxicity of artemisinin on H69VP cells (IC_{50} = 24 nM) was ten-fold lower than for H69 cells (IC_{50} = 2.3 nM). Pretreatment with ~880 nM holo-transferrin did not alter the cytotoxicity of artemisinin on H69 cells, but significantly enhanced the effect on H69VP cells (IC_{50} = 5.4 nM) (Sadava et al., 2002).

Efferth and associates also reported that Fe(II)-glycine sulfate and transferrin enhanced the cytotoxicity (~10.3-fold) of free artesunate, artesunate microencapsulated in maltosyl-β-cyclodextrin, and artemisinin towards CCRF-CEM leukemia and U373 astrocytoma cells (Efferth et al., 2004).

Dihydroartemisinin is also cytotoxic to human glioma cells (U373MG), and the sensitization is markedly enhanced by the addition of holo-transferrin (Kim et al., 2006). In addition, radiation-induced expression of the endogenous antioxidant enzyme glutathione-*S*-transferase was found to be suppressed by dihydroartemisinin (Kim et al., 2006).

These in vitro data indicate that artemisinin derivatives kill or inhibit the growth of many types of cancer cell lines, including drug-resistant cell lines, suggesting that artemisinin could become the basis of a new class of anticancer drugs. Also, addition of holo-transferrin and other iron sources clearly increase the potency of artemisinin in killing cancer cells.

Angiogenesis, the proliferation and migration of endothelial cells resulting in the formation of new blood vessels, is an important process for the progression of tumors. Antiangiogenesis effects of artemisinins have also been reported.

Artesunate has been shown to inhibit the growth of Kaposi's sarcoma cells, a highly angiogenic multifocal tumor, and that the activity of cell growth inhibition correlated with the induction of apoptosis (Dell'Eva et al., 2004). Treatment with artesunate at more than 2.5 μM for 48 h inhibited the proliferation of human vein endothelial cell (HUVEC) in a concentration-dependent manner by the MTT assay ($p < 0.05$) (Chen et al., 2004a). The IC_{50} value was 20.7 ± 3.2 μM and HUVEC can be inhibited by 88.7% by 80 μM of artesunate (Chen et al., 2004a).

5. In vivo anticancer bioactivity of artemisinins

Moore et al. reported that oral administration of dihydroartemisinin and ferrous sulfate inhibited the growth of implanted fibrosarcoma tumors in the rat (Moore et al., 1995). The growth rate of the tumors was retarded (30% less than control group) by daily oral administration of ferrous sulfate (20 mg/kg/day) followed by dihydroartemisinin (2–5 mg/kg/day), and no significant tumor growth retardation effect was observed in the administration of either dihydroartemisinin or ferrous sulfate alone (Moore et al., 1995).

Lai and Singh (2006) have also reported the potential of artemisinin to prevent breast cancer development in rats treated with 7,12-dimethylbenz[*a*]anthracene (DMBA), a carcinogen known to induce multiple breast tumors. Prevention from developing breast cancer was shown in 43% of DMBA-treated rats fed artemisinin for 40 weeks, whereas almost all the rat fed normal food developed tumors within that time. Breast tumors of artemisinin-fed rats were also significantly fewer and smaller in size compared with control (Lai and Singh, 2006).

The antiangiogenic effect of artemisinin was also studied in nude mice implanted with human ovarian cancer HO-8910 cells (Chen et al., 2004a). With artesunate treatment, tumor growth was decreased and microvessel density was reduced with no apparent toxicity to the animals (Chen et al., 2004a). Tumor growth in rats given 50 mg/kg/day and 100 mg/kg/day of artesunate subcutaneously for 15 days was reduced by 41% or 62%, respectively (Chen et al., 2004a). Microvessel density as a parameter of angiogenic activity in the tumors of animals treated with 100 mg/kg of artesunate daily was at least four times lower than in the control group (Chen et al., 2004a). In a model of chicken chorioallantoic membrane (CAM) neovascularization, the antiangiogenic activity was also evaluated, and dihydroartemisinin significantly inhibited CAM angiogenesis at low concentration (~30 nmol/100 μl per egg) (Chen et al., 2004b).

Human cancers have been treated successfully with artemisinin derivatives. These studies encourage further investigation of artemisinin in human cancer cases under well-controlled clinical studies (Berger et al., 2005; Singh and Verma, 2002; Singh and Panwar, 2006).

6. Molecular mechanisms of artemisinin's anticancer action

Artemisinin and derivatives induce apoptosis in cancer cells (Li et al., 2001; Chen et al., 2004b; Singh and Lai, 2004; Wu et al., 2004). Mercer et al. (2007) have reported that dihydroartemisinin induces concentration- and time-dependent mitochondrial membrane depolarization and activation of caspases-3 and -7 in HL-60 cells. Artemisinin derivative induces apoptosis in P388 murine leukemia cells mainly during the G1 phase of the cell cycle (Li et al., 2001). This is understandable since enhanced transferrin receptors expression and increased iron uptake occur during G1 phase. Efferth et al. (2004) reported that after application of 3 μg/ml of artesunate to CCRF-CEM leukemia cells in combination with Fe(II)-glycine sulfate, cell

cycle perturbations were not observed. However, the fraction of apoptotic cells increased up to 33% after 96 h and the percentage of cells in G_0/G_1 phases decreased. From these data, the major fraction of apoptotic cells was conceivable to be from this cell cycle phase.

Down-regulation of the bcl-2 protein and up-regulation of bax protein in artesunate-treated human umbilical vein endothelial cells were shown by Western immunoblot analysis (Wu et al., 2004). From these analyses, pro-apoptotic bax expression was up-regulated in a concentration-dependent (8–32 μM) and time-dependent (6–24 h) manner. On the other hand, anti-apoptotic bcl-2 expression was down-regulation synchronously (Wu et al., 2004).

Artesunate and dihydroartemisinin lowered vascular endotherial growth factor (VEGF) expression and VEGF receptor KDR (kinase insert domain containing receptor)/flk1 (fms-like tyrosine kinase) expression on tumor cells (Chen et al., 2004a,b). VEGF has been known to be a potent angiogenic factor. It binds to VEGF receptor on endothelial cells surface and activates various functions of angiogenesis. VEGF can also be secreted by many human tumors. When mice, implanted with HO-8910 cells, were injected with ∼100 mg/kg artesunate, significantly weaker VEGF expression on the tumor cells was observed. Also artesunate could reduce KDR/flk1 expression in tumor cells or endothelial cells by 70 or 80% (Chen et al., 2004a).

The mechanism of cytotoxicity induced by artemisinins seems to involve many different pathways, and more studies are needed to understand it.

7. Targeted drug delivery using transferrin

As described above, cancer cells need iron uptake by the transferrin-mediated mechanism to maintain their uncontrolled growth, and transferrin receptors are highly expressed on cancer cell surface. Transferrin has been used as a drug-carrier to target cancer cells (Li and Qian, 2002; Qian et al., 2002).

One example is doxorubicin linked with transferrin via the formation of a Schiff base maleimide spacer. The transferrin-doxorubicin conjugate has been shown to exhibit a greatly increased cytotoxicity, compared to doxorubicin alone, toward a variety of culture cell lines, including many multidrug-resistant cancer cells (Fritzer et al., 1992, 1996; Sizensky et al., 1992; Singh et al., 1998). In vivo studies also showed that the life span of tumor-bearing mice was significantly increased by treatment with transferrin–doxorubicin conjugate, and the tumor burden of conjugate-treated mice was much smaller compared with doxorubicin-treated mice (Singh et al., 1998).

Transferrin has also been used for gene delivery. For the efficient introduction of genes into many cell lines, polylysine-, polyethyleneimine-, cationic liposome-transferrin conjugates have been shown to be efficient carriers (review Qian et al., 2002).

Thus, therapeutic agents can be chemically conjugated to transferrin for their specific delivery to cancer cells. These conjugates are generally made by the modification of amino acids moieties of transferrin with the agents, which may affect the functions of transferrin such as its ability to bind to receptor. Also within an endosome, release of the agents from transferrin may be an issue because of the covalent conjugations. These problems should be considered for more efficient drug delivery using transferrin.

8. Artemisinin-tagged to holo-transferrin via carbohydrate chains of the protein

Transferrin has two N-glycosides attached to Asn residues in the C-terminal (Asn-413 and Asn-611) (Gomme et al., 2005). These carbohydrate chains are not involved in receptor binding of transferrin (Mason et al., 1993). In equilibrium binding experiments, nonglycosylated mutant of transferrin could bind to HeLa S_3 cells with the same avidity as glycosylated transferrin (Mason et al., 1993). Irie et al. (1988) also reported that treatment with double-labeled transferrin (sialic acids of carbohydrate chains with ^{3}H and protein core with ^{125}I or ^{59}Fe) on liver endothelium led to a concordant cellular uptake of the two labels initially. Then these labels were dissociated and ^{3}H was mainly retained in the cells. From these data, carbohydrate chains seem to be an ideal place to attach therapeutic agents to transferrin.

Lai et al. (2005a,b) reported the transportation of both iron and artemisinin into cancer cells by covalent tagging of artemisinin analogs to the N-glycoside moiety of holo-transferrin (Fig. 2). Holo-transferrin was reacted with periodate to oxidize the N-glycoside chains (sialic acid and 1,2-diol) to expose aldehyde groups on the protein surface, and then artelinic acid hydrazide was reacted with the oxidized transferrin to form a covalent conjugate. The artemisinin-tagged transferrin was tested on Molt-4 and normal human lymphocytes. LC_{50}s, as determined by the probit analysis (dose-response at 72 h of treatment), were 0.98 μM and 33 mM for Molt-4 and normal human lymphocytes, respectively. The artemisinin-tagged transferrin was 34,000 times more potent in killing Molt-4 cells than its normal human lymphocytes. In the case of dihydroartemisinin, it was 36 times more potent in killing Molt-4 cells than lymphocytes in same assay. Furthermore, holo-transferrin with artemisinin tagged to lysine residues of the protein was less effective in killing Molt-4 cells than holo-transferrin with artemisinin tagged to carbohydrate chains (Lai et al., 2005a). Presumably, tagging to the protein moiety affected the binding of transferrin to its receptors.

We also have optimized the tagging reaction of artemisinin analogues and transferrin via the N-glycoside chains, and were able to control the average number of artemisinin moieties per protein molecule by changing conditions for the oxidation and tagging steps. Holo-transferrin tagged with an average of 16 molecules of artemisinin, showed only minimal structural change, could kill prostate cancer cells effectively through the receptor-mediated endocytosis (paper in preparation). Also, the artemisinin-tagged transferrin could efficiently induce apoptotic cell death via cytochrome *c* release from mitochondria, caspases activation, and DNA degradation (paper in preparation). These results suggest that tagging of drugs to the N-glycoside chains of transferrin provides a very effective mean of targeted delivery of drugs into cancer cells.

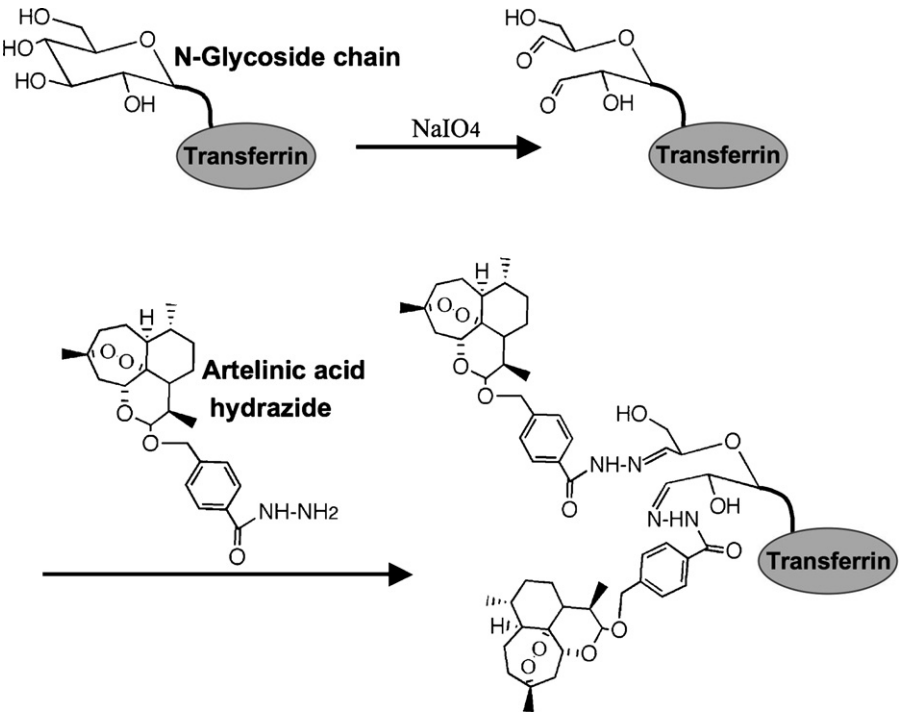

Fig. 2. Synthesis of artemisinin-tagged transferrin via carbohydrate chains.

9. Conclusion

Artemisinin and its derivatives have very unique chemical structure and reactivity. Unlike other peroxide compounds, artemisinin can be stored at room temperature for many years without noticeable decomposition. The endoperoxide core can withstand a variety of reagents including some reducing agents such as hydrazine and $NaBH_4$, allowing the synthesis of an array of semi-synthetic artemisinins, with the same reactivity with Fe(II). Its selective cytotoxicity toward cancer cells, as reported in several papers, is encouraging for its development into an effective low-cost cancer therapy. Furthermore, tagging of molecules to the carbohydrate chains of transferrin is a better approach for targeted delivery of therapeutic agents into cancer cells via the transferrin receptor mechanism. Artemisinin is particularly suitable for this purpose, since artemisinin only becomes toxic after reacting with iron. Thus, artemisinin-tagged transferrin brings both a prodrug (artemisinin) and it's activating agent (iron ion) into a cell, and artemisinin is activated and becomes toxic only inside the target cell, where iron is released and reacts with it.

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