Targeted treatment of cancer with artemisinin and artemisinin-tagged iron-carrying compounds

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Artemisinin is a chemical compound that reacts with iron to form free radicals which can kill cells. Cancer cells require and uptake a large amount of iron to proliferate. They are more susceptible to the cytotoxic effect of artemisinin than normal cells. Cancer cells express a large concentration of cell surface transferrin receptors that facilitate uptake of the plasma iron-carrying protein transferrin via endocytosis. By covalently tagging artemisinin to transferrin, artemisinin could be selectively picked up and concentrated by cancer cells. Furthermore, both artemisinin and iron would be transported into the cell in one package. Once an artemisinin-tagged transferrin molecule is endocytosed, iron is released and reacts with artemisinin moieties tagged to transferrin. Formation of free radicals kills the cancer cell. The authors have found that artemisinin-tagged transferrin is highly selective and potent in killing cancer cells. Thus, artemisinin and artemisinin-tagged iron-carrying compounds could be developed into powerful anticancer drugs.

Keywords: artemisinin, cancer, iron-carrying molecule

1. Introduction

In 1972, artemisinin was discovered to be the active ingredient responsible for the antimalarial action of the Chinese medicinal herb qing-hao (Artemisia annua L.), which has been used in China for centuries for afflictions such as fevers, haemorrhoids, and malaria. Artemisinin has a strong effect on chloroquine-resistant cases of malaria and shows no evidence of serious toxicity [1,2]. To date, artemisinin and its analogues have been used for the treatment of > 2 million cases of malaria infection.

Artemisinin is a sesquiterpene lactone peroxide containing an endoperoxide moiety (-O-O-C-) which forms free radicals when it reacts with iron [3]. This carbon-based radical, when formed in cells, can lead to cellular damage and cell death by reacting with cellular macromolecules such as proteins and membrane lipids. Because malaria parasites contain large amount of haem-iron, a product from the digestion of haemoglobin within host red blood cells, a hypothesis is that interaction of artemisinin with haem leads to death of the parasite [4,5]. However, a recent experiment [6] indicates that activation of artemisinin inside the parasite is by ferrous iron independent of haem. The source of the iron is not known.

The pharmacology and pharmacokinetics of artemisinin and some of its analogues have been well-studied and documented [7-16]. Various analogues of artemisinin and endoperoxide-containing compounds have been synthesised and some are being used in malaria treatment [17-19]. Figure 1 shows the chemical structures of artemisinin and two analogues, dihydroartemisinin (DHA) and arteletic acid.

The rationale of the targeted therapy discussed in this paper is based on a concept that takes advantage of the iron-catalysed free radical-forming property of artemisinin and argues it toward cancer cells.
2. Artemisinin, iron and cancer cells

Because iron plays a vital role in cell physiology and growth (e.g., in energy metabolism and DNA synthesis, as iron is a cofactor of the enzyme ribonuclease, which converts ribose to deoxyribose), special molecular mechanisms have evolved for the transport of iron into cells. In vertebrates, a major iron transport system involves a specific interaction between the iron-carrying protein transferrin, in the plasma, and cell surface transferrin receptor, that results in a facilitated transport of iron into the cell via endocytosis [28].

Iron, in its free form, is toxic because it catalyzes the formation of free radicals; notably, formation of a hydroxyl radical from hydrogen peroxide (the Fenton reaction) that is generated as a metabolic by-product in the mitochondria. Most iron in cells is rendered inactive by chelating to other molecules or stored in ferritin as iron oxide. Iron is transported in the blood by binding to transferrin. Cells in need of iron express transferrin receptors on their surface. Binding of holotransferrin (i.e., iron-loaded transferrin) to the receptor, triggers endocytosis and transports holotransferrin inward in intracellular endosomes. Once the pH is inside an endosome droplet, iron is released in the ferrous form and actively transported from the endosome into the cytoplasm. Apotransferrin (iron-free transferrin) remains attached to the receptor at low pH and is recycled to the cell surface and released. Released iron is immediately used in cellular processes, for example, incorporation into enzymes and cytochromes. Excess iron is converted into an oxide form and stored in ferritin. Feedback mechanisms, involving transcription and post-transcription processes, are involved in iron metabolism and maintain intracellular iron in a tightly controlled state.

Due to their rapid rate of division, most cancer cells have high rates of iron intake [21] and express a high cell surface concentration of transferrin receptors [22] than normal cells. This occurs during the G1 and S phases of the cell cycle to prepare the cell for DNA replication and subsequent mitosis. In general, the aggressiveness of a tumor is positively correlated with transferrin receptor concentration and the proliferation index of its cells. As cancer cells have a higher iron influx via the transferrin receptor mechanism, cancer cells would be more susceptible to the cytotoxic effect of artemisinin. This concept suggests that artemisinin could be effective for the treatment of many types of cancer.

Many examples of increased requirement and dependency on iron by cancer cells to proliferate can be found in the research literature. The following is a description of several common types of cancer. For example, breast cancer cells have 5–15 times more transferrin receptors on the cell surface than normal breast cells [28]. Transferrin receptors are overexpressed only on the cell surface of breast cancer cells, but not on benign breast tumour cells [23]. More importantly, it has been shown that breast cancer cells do take up more iron than normal breast cells [25]. In addition, the dependence of breast cancer cells on iron intake is suggested by the finding that antibody to transferrin receptors can retard the growth of breast tumour [26]. Artemisinin has been shown to be selectively toxic to human breast cancer cells [27]. Iron also plays an important role in brain tumour cell proliferation, particularly involving the transferrin iron uptake mechanism. For instance, transferrin receptors are present in high concentrations on the cell surface of glioblastoma and meningiomas [28–30]. Transferrin has been shown to be endocytosed into neuroblastoma cells [31]. Cancer cells that metastasised to the brain also express high concentrations of transferrin receptors [32]. There is a positive correlation between transferrin receptor expression and Ki-61 growth fraction in glioma [33]. Artemisinin and its analogues have been shown to be toxic to brain cancer cells [34]. Artemisinin analogues have been shown to inhibit proliferation of undifferentiated neuroblastoma (N32A) and glioma (C6) cells [34] and glioblastoma multi-forme cell line [35]. In chronic myelogenous leukaemia (CML), cells also express more transferrin receptors on their cell surface than normal cells [36–38]. In addition, the dependence of CML cells on iron intake via the transferrin mechanism is suggested by the finding that antibody to transferrin receptors retards the growth of both CML cells [39–42] and conjugates of transferrin with cytotoxic agents are effective at killing CML cells [43–46]. Artemisinin is selectively toxic to human leukaemia cells [40]. Leukaemia cells have been shown to be the most sensitive to artemisinin, an artemisinin analogue, among various other cancer cells [41].
Thus, because of this increased requirement of iron by cancer cells, artemisinin would be selectively toxic to cancer cells and constitutes a targeted-therapy for cancer.

3. Artemisinin and cancer: experimental results

The authors have shown that MOLT-4 cells, a human leukaemia cell line, are more susceptible to the cytotoxic effect of artemisinin than their normal counterparts, (i.e., normal human lymphocytes) [50]. The LC₅₀ (lethal concentration)₅₀ of artemisinin for MOLT-4 cells is ~ 100 times lower than that of lymphocytes. An important observation in this experiment is that addition of holotransferrin to the cell culture significantly enhanced the toxicity of artemisinin to cancer cells, suggesting the involvement of intracellular iron in the effect. Further research in the authors’ laboratory has shown that artemisinin induces mainly apoptosis in MOLT-4 cells [50].

In another experiment, the authors tested artemisinin on two human breast cell lines [27]: HTB 125, a normal human breast cell line with epithelial cell morphology, and HTB 27, a radiation-resistant human breast cancer cell line, also with epithelial cell morphology. The authors found that only 2% of breast cancer cells were still alive after a 16-hour treatment with both DHA and holotransferrin. On the other hand, treatment with DHA alone or DHA + holotransferrin had little effect on normal human breast cells. These data indicate that artemisinin is selectively toxic to the radiation-resistant human breast cancer cell line HTB 27, but not to normal breast cells. Furthermore, it was found that holotransferrin significantly enhanced the cytotoxicity of artemisinin on breast cancer cells. Thus, iron plays an important role in the effect as the authors hypothesized. More recently, Effertz et al. showed that ferrous iron enhanced the cytotoxic effect of artemisinin toward leukaemia and astrocytoma cells [62].

Other research indicating the role of iron is an in vivo study carried out by the authors in which rats implanted with fibrosarcoma were treated with DHA. The authors found that administering iron-ferric-sulphate orally to the rats enhanced the effect of DHA in reducing tumour growth (oral iron supplement could increase the concentration of holotransferrin in the plasma, because normally only 20% of the transferrin in the blood carries iron). However, in a similar experiment on implanted breast tumours in the rat, the authors found iron administration did not significantly affect the effect of artemisinin (unpublished data).

In addition to the authors’ findings, other researchers have also reported the potential selective antitumour properties of artemisinin and its analogues both in vitro and in vivo on various forms of cancer [51,64-66]. There are also two case reports of patients with large-cell squamous cell carcinoma (57) and metastatic uterine melanoma (56) treated with artemesinin.

Artemisinin has also been shown to impede angiogenesis [51,52,67-74]. This may be another mechanism of the anticancer effect of artemisinin in vivo. The antiangiogenesis effect has been shown in chicken choioallantoic membrane, human ovarian tumour implanted in nude mice, mouse embryonic stem cell-derived embryoid bodies and tube formation of human umbilical vein endothelial cells. The effect apparently involves downregulation of vascular endothelial growth factor-related processes and free radicals.

Artemisinin is a relatively safe drug with no obvious adverse reactions or noticeable side effects [27,51-74]. This is supported by the above in vivo experiments in which no obvious toxic reactions in normal cells was observed. Nonetheless, it must be pointed out that neurotoxicity has been reported with some analogues of artemisinin. For example, intramuscular administration of artemether at 20 mg/kg/day for 8 days, has been reported to induce neurological deficits in the dog, and artemether at 12.5 - 50 mg/kg/day for 28 days, also induced neurological syndromes in the rat [75]. However, oral and subcutaneous administrations of the synthetic analogue Ro42-1611 (artefranic) to the rat at 400 mg/kg/day for 4 weeks were well-tolerated and did not induce any mutagenic effect [76]. Existing data seem to indicate that oral artemisinin is relatively safe and causes no side effects [76].

The safety of artemisinin in vivo is born out in a recent experiment that the authors performed to study the use of artemisinin for cancer prevention [66]. In the experiment, the effect of daily oral artemisinin intake on preventing the development of breast cancer in the rat was studied. Rats were treated with the carcinogen 9,10-dimethyl-1,2-benzanthracene (DMBA) that is known to induce multiple breast tumours. After DMBA treatment, some rats were provided with a powdered rat-chow mixed with 0.02% artemisinin, whereas other animals (controls) were provided with the powdered food without artemisinin. The daily oral intake of artemisinin of the experimental animals was estimated to be 8 ± 10 mg/kg.

Development of breast cancer in the rats was monitored for 40 weeks. Data show that oral artemisinin significantly delayed (i.e., artemisinin-fed rats that developed breast cancer took significantly longer time for the first tumour to appear) and prevented (i.e., significantly more of the artemisinin-fed rats did not develop cancer within 40 weeks after carcinogen treatment) the development of breast cancer in the rat. In addition, artemisinin-fed rats developed significantly fewer numbers of tumours, and the total volume of tumours in an animal was significantly smaller. These data indicate that daily oral intake of artemisinin is effective in the prevention of breast cancer. Even after 40 weeks of daily oral intake of artemisinin, no apparent health effect was observed in animals that did not develop cancer.

4. Artemisinin-tagged transferrin

As mentioned above, in mammalian cells, iron is transported into cells via a receptor-mediated endocytosis process [20]. Binding of the plasma iron-carrying protein holotransferrin to cell surface transferrin receptors triggers endocytosis that
Figure 2. Structure of diametanoyl saccharide of transferrin.

transfers transferrin and the iron it carries into cells. Because cancer cells require a large amount of iron, they express a high number of transferrin receptors on their surface and take up more iron during G1 and S phases of the cell cycle. The authors speculate that if artemisinin is cotransported to holotransferrin, it would be transported as a complex into cells and react with iron within the endosome where iron is 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. Transferrin has two N-glycans attached to the asparagine residues in the C-terminal domain. Periodate oxidation of these carbohydrate chains generates reactive aldehyde groups that can be modified with a variety of hydrazine or amine derivatives of artemisinin. Thus, the artemisinin analogue artemetic acid has been tagged to the glycosyl-moiety of holotransferrin using a relatively simple process. The authors estimate that as many as 10 artemisinin derivatives could be tagged to a molecule of transferrin. The following is a brief description of the procedures that may be used to synthesize artemisinin-tagged transferrin.

4.1 Method of attaching artemisinin to transferrin

The two N-glycans attached to the asparagine residues in the C-terminal domain of a transferrin molecule are not involved in binding of transferrin to its receptor. Periodate oxidation of these carbohydrate chains generates reactive aldehyde groups that can react with hydrazine or amine derivatives of artemisinin. The carbonyl group of transferrin is a primary aminesaccharide (Figure 2). Assuming that all 1,2-diol moieties are oxidized to the corresponding aldehydes, the authors estimate that > 10 artemisinin units could be tagged to a molecule of holotransferrin. Artemisinin has also been tagged to amino acid side chains, such as lysine, on the protein surface. Such lysine-based tagging strategy could, however, interfere with receptor binding. The authors have shown that carbohydrate-based tagging chemistry produced a more potent tagged protein compared to lysine-based tagging chemistry.

Artemetic acid was used for the tagging. Artemetic acid has a rigid spacer to keep the bulky artemisinin moiety away from the carbohydrate group. Artemetic acid methyl ester was firstly synthesized, and then reacted with hydrazine to produce the desired artemetic acid hydrazone (ART-PH-NH2) (Figure 3).

Holotransferrin was then oxidized by sodium periodate in acetate buffer, pH 5.5, for 30 min. Excess reagent and by-products were removed by gel filtration chromatography on Sephadex G-25. The protein fractions were combined, and immediately reacted with artemetic acid hydrazone to produce the tagged transferrin (Figure 4). The product was purified by the second gel filtration column (Sephadex G-25) that had been equilibrated with saline buffer, pH 7.5. The protein fractions were collected and stored at 4°C. Unlike oxidized transferrin, tagged protein was found to be very stable, and can be stored at 4°C for at least 12 months without any significant change in its biological activities.

Ultraviolet/visible and circular dichroism spectra of artemisinin-tagged transferrin showed that the protein structure remained largely intact during the protein modification. However, the iron content decreased from ~100 to 40% during the periodate oxidation step. The iron content remained unchanged during the tagging step. Periodate, therefore, damages the iron binding site, probably by oxidizing some coordinating amino acid residues such as Tyr and His. Procedures to prevent this damage are now being developed in the authors' laboratory.
92 LI J, FENG J, FAN YR et al: Protein ubiquitination of antilysostatin by anabolic action of an agonist containing a transferrin-sapinephrine.

Figure 3. Synthesis of artemisinic acid hydrazine.

Figure 4. Chemical modification of human transferrin with artemisinin.

Hydrophilic interaction high performance liquid chromatography (HPLC) was used to determine the purity of the tagged protein. Because artemisinin is a hydrophilic compound, a protein- transferrin-protein mixture was prepared on the HPLC condition. The peak of tagged transferrin was much broader than native transferrin, suggesting that the authors’ sample was a mixture of tagged proteins with different numbers of artemisinic acid moieties on the protein surface. Matrix-assisted laser desorption-ionization mass spectrometry confirmed the HPLC data. Tagged transferrin and native transferrin gave their molecular ion peaks at 77,619 Da and 75,828 Da, respectively. The mass difference corresponded to the tagging of 4.2 artemisinic moieties per protein. The peak shape of tagged protein was again, much broader than that of native transferrin.

The authors were able to show that artemisinin moieties in tagged protein contain the intact endoperoxide bond. Both DHA and artemisinin produce chemiluminescence when they add to hematin and luminal due to their endoperoxide bond. When tagged protein was reacted with the chemiluminescence receptor (hematin or luminal), the solution produced a time-dependent chemiluminescence, similar to that of dihydroartemisinin.

4.2 Effect of artemisinin-tagged transferrin on cancer cells

The authors tested this artemisinin-tagged transferrin (tagged transferrin) on the human leukemia cell line MOL-4 and normal human lymphocytes. The potency of
Targeted treatment of cancer with arsenitmin and arsenitmin-tagged iron-carrying compounds

Lai, Sasi & Singh

1. Introduction

The following is the COG view of the ‘treated-to-respond’ DHA on Multi-4 cells and lymphocytes, as determined by the Probit analysis: Multi-4-compound 0.98 μM, Multi-4-DHA 1.6 μM, and Multi-4-tagged compound 43 μM; lymphocytes 0.8 μM. Thus, compared with DHA, the ‘treated-to-respond’ is more potent in killing Multi-4 cells and less potent in killing normal lymphocytes. DHA is not shown to develop resistant sublines in a small number of assays, and this suggests that there is a higher concentration of the compound in patients with advanced cancer than in normal lymphocytes (unpublished data).

The half-life of plasma transferrin in normal human subjects has been estimated to be 11 ± 0.3 days [10]. A concern might be the possibility of an increase in serum transferrin levels in cancer patients, but this is not expected to be a problem. However, the presence of conjugates, which might be lower than the compound, in cancer patients, has to be considered. Moreover, it is not expected to be a significant problem.

2. Conclusion

A major consideration in effective drug therapy is selectivity, such that the dose of drug needed for the desired effect would be reduced. However, it is conceivable that in some cases, the desired effect might be reduced, which could be detrimental to the patient. For example, the desired effect of a drug might be reduced if the drug interacts with other drugs, which could be problematic.

Further advantages of using arsenitmin for cancer treatment are that it is effective orally and is not ionized. In addition, the interaction of arsenitmin with the Akt pathway, which is involved in cancer cell survival, is being developed to genetically engineer bacteria to synthesize arsenitmin, which could then be converted to arsenitmin by modified cells.

As noted in the previous section, arsenitmin provides a further improvement in selectivity. In this case, arsenitmin is selectively brought into cancer cells, and release of iron from the transferrin molecule immediately triggers the cytoaxic action. Targeted transferrin to transferrin also increases the half-life of arsenitmin in the body [17]. The short half-life of arsenitmin and its analogues [18] has been a problem in their therapeutic effectiveness, as the concentration of the compound in a natural compound and iron is an important component in cellular functions, it is less likely that tolerance would be developed to arsenitmin-transferrin transfer. However, the anticancer effectiveness of arsenitmin-transferrin is in vivo remains to be studied.

6. Expert opinion

Arsonitmin is an effective anticancer compound that produces few side effects. Because it is inexpensive and effective orally, it could be useful as an anticancer agent for patients in developing countries. While this drug is not suitable for all patients, it is likely that more studies are needed to determine if the compound could be found to be safe.

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**A thorough review of cellular iron metabolism.**


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50. CHOWDHURY NR, BAINES M, JONGH T, DAVIN Y, FRIED G, LAMB B, ISHIO K. The effect of artemisinin was studied in both breast cancer cell and normal breast cells at log phase when they were dividing at comparable rates. It was found that artemisinin was toxic to the cancer cells and not the normal cells. Why are dividing normal cells less sensitive to artemisinin? One possibility is that iron metabolism is less well-controlled in cancer cells. Thus, more free iron is available and can act with artemisinin. It has been reported that some cancer cells express plasmal-like isoenzymes, which is less effective than ferritin in storing iron. On the other hand, it is also possible that cancer cells intake iron at a faster rate than dividing normal cells, thus, increasing the chance of spill over. As this selectivity is also true with artemisinin-targeting transferrin, it is...
possible that endosomes in cancer cells are less effective in transporting released iron out to the cytoplasm.

One possible drawback of artemisinin-targeted transferrin is that it is likely to compete with endogenous transferrin for binding to transferrin receptors [18]. A possible method to overcome this is to increase the tigogen efficiency such that more artemisinic moieties would be attached to a transferrin molecule of transferrin. This has to be done such that the tagged iron does not affect the affinity of the transferrin to its receptor.

By manipulating the transferrin-receptor iron-transport system advantageously, the efficiency of artemisinic and artemisinin-targeted transferrin can be enhanced. As transferrin receptors on the cell surface are coupled to other membrane components, means can be applied to modify the rate of iron delivery into cells. For example, the K62 cell line has been widely used as an in vitro model for CMV. Various compound have been shown to enhance iron content in K62 cells [18]. K62 cells have been shown to upregulate surface transferrin receptors, thus enhancing iron uptake in K62 cells [18]. Erythropoietin also increases transferrin receptor expression and intracellular iron content in K62 cells [18]. Thapsigargin has been shown to speed up calcium-dependent iron uptake and increase the overall capacity of K62 cells in taking iron by accelerating the transferrin receptor endocytosis process [18]. Conceivably, these compounds could enhance the potency and specificity of artemisinin for the treatment of CMV. Another interesting membrane component is the receptor for insulin-like growth factor I (IGF-I), which is expressed in a wide variety of cells. IGF-I has been shown to enhance iron influx into cells, partially by reducing the rate of endocytosis, and allows more time for cell surface transferrin receptors to pick up transferrin. Furthermore, IGF-I receptors are upregulated in higher expression in cancer cells than in normal cells [18]. This rise in IGF-I and artemisinin-targeted transferrin could further enhance the potency and selectivity of the targeted-compound in killing cancer cells. Because artemisinin works as a free radical, its effectiveness could also be enhanced by increasing oxygen tension, decreasing intake of antioxidants, and blockade of proinflammatory and cytokine reactions such as monocyte recruitment to the site of oxidative stress [18]. However, these treatments are not to be tried since they would not also increase the uptake of artemisinin towards normal cells.

Due to the emergence of diverse drug-resistant bacteria, there is an urgent need to develop new antibiotics [12]. Some of these drug-resistant bacteria, for example, Staphylococcus aureus, Streptococcus pneumoniae, Pseudomonas aeruginosa, and Mycobacterium tuberculosis, are major problems of human health, particularly in developing countries.

Because iron is an essential nutrient for growth, development and cellular functions, living organisms have evolved sophisticated mechanisms to obtain iron from the environment [114-117]. A strategy used by some bacteria is to hijack iron from iron-containing compounds endogenous to their hosts. For example, certain bacteria (see Table 2) have receptors on the surface to bind host transferrin and lactoferrin (an iron-carrying protein) that are abundant in body fluids such as mucus. After binding, iron is released and actively transported into a bacterium [120]. These bacterial iron assimilation mechanisms can provide a basis for effective drug targeting and delivery.

Studies have shown that, in general, a high concentration of artemisinin is required to kill bacteria [120]. This is probably because iron is very tightly regulated at the bacterial level. Artesinin is ineffective without iron. The question is whether or not artemisinin-targeted transferrin and lactoferrin are effective in killing bacteria that utilize these iron-transport proteins. The preliminary data shown have that artemisinin-targeted transferrin is quite effective in inhibiting the growth of M. tuberculosis compared with sonicated M. tuberculosis has been shown to use transferrin and lactoferrin and iron to obtain iron [112]. Artemisinin could also be tagged to other physiological iron-carrying molecules. For example, bacteria have two basic mechanisms to pick up iron from the environment.

The first is to hijack iron from iron-containing compounds endogenous to their hosts, such as transferrin and lactoferrin, as discussed above. Another mechanism is secretion and recombination of highly specific iron-binding chemicals known as siderophores. Two major forms of bacterial siderophores are catechol and hydroxamate that have very high iron affinity. They are synthesized and secreted by certain bacteria to scavenge iron from the environment. Bacteria have developed specific receptors to bind and pick up siderophores and transport them inside through molecular channels. Once inside, iron is released from the siderophores. Examples of bacteria utilizing the siderophore mechanism are Escherichia coli, Salmonella typhimurium, Klebsiella pneumoniae and P. aeruginosa. There are also bacteria that use both iron-carrying proteins and siderophores. Examples are E. coli and M. tuberculosis. Other bacteria (e.g., Campylobacter jejuni) that do not produce siderophores, but pick up siderophores released by other bacteria. By the same token as the artemisinin-targeted transferrin, artemisinin can be tagged to siderophores and these compounds may be useful for treatment of bacterial infection. Tagging artemisinin to siderophores is relatively easy. Because the molecules are small, they can be administered orally. Also, as mammalian cells do not bind or reuptake iron, they should be non-toxic to humans. Miller and co-workers have explored the receptor-mediated transport of siderophores to deliver antibacterial agents, by tagging J1-lucerna, 5-fluorouracil and other antibiotic compounds to siderophores [121-124]. These compounds kill bacteria at mid-to-high micromolar concentrations. Thus, the use of artemisinin-targeted siderophores for treatment of bacterial infection is feasible. Again, they provide the advantage and efficiency of bringing both artemisinin and its targeting agent into physiological tissues simultaneously. It also should be pointed out that certain fungi also use siderophores to obtain iron [116]. Artemisinin-targeted siderophores may, therefore, also be useful for the treatment of fungal infection.

In general, compounds that contain iron-sensitive endoperoxide and physiological iron-carrying compounds tagged with endoperoxide, fit into the scheme of targeted therapy discussed in this paper.

Table 2. Receptors for iron-carrying proteins in some human pathogens.

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Disease</th>
<th>Receptor</th>
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<tbody>
<tr>
<td>Moraxella catarrhalis</td>
<td>Otis media</td>
<td>Transferrin, lactoferrin</td>
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<td>Moraxella lacunata</td>
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<td>Transferrin, lactoferrin</td>
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<td>Meninges</td>
<td>Transferrin, lactoferrin</td>
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<td>Zoonotic enteropathogen</td>
<td>Transferrin, lactoferrin</td>
</tr>
<tr>
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<td>Meningitis, otis media</td>
<td>Transferrin, lactoferrin</td>
</tr>
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<td>Transferrin, lactoferrin</td>
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<td>Transferrin, lactoferrin</td>
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<td>Pneumonia, meningitis, bacteria, otis media</td>
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<td>Gastritis, gastric and duodenal ulcers, gastric adenocarcinoma, lymphoma</td>
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</table>

Bibliography

Papers of special note have been highlighted as either (***) or of considerable interest (**) to readers.

Targeted treatment of cancer with artemisinin and artemisinin-tagged iron-carrying compounds

Lai, Sasaki & Singh

Table 2. Receptors for iron-carrying proteins in some human pathogens.

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Disease</th>
<th>Receptor</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Mesorillia catarrhalis</em></td>
<td>Oral cavity disease</td>
<td>transferrin, lactoferrin [121]</td>
</tr>
<tr>
<td><em>Moraxella lacunata</em></td>
<td>Kerato-conjunctivitis</td>
<td>transferrin, lactoferrin [121]</td>
</tr>
<tr>
<td><em>Neisseria meningitidis</em></td>
<td>Meningitis</td>
<td>transferrin, lactoferrin [121]</td>
</tr>
<tr>
<td><em>Neisseria gonorrhoeae</em></td>
<td>Gonorrhea</td>
<td>transferrin, lactoferrin [121]</td>
</tr>
<tr>
<td><em>Actinobacillus actinomycetemcomitans</em></td>
<td>Jaw periodontal disease</td>
<td>transferrin, lactoferrin [121]</td>
</tr>
<tr>
<td><em>Haemophilus influenzae</em></td>
<td>Meningitis, oral media</td>
<td>transferrin, lactoferrin [121]</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>Bacteremia, pneumonia, endocarditis, septic arthritis, osteomyelitis, deep abscesses, food poisoning</td>
<td>lactoferrin, transferrin [121]</td>
</tr>
<tr>
<td><em>Staphylococcus epidermidis</em></td>
<td>Endocarditis, endophthalmitis, septicaemia, cellulitis</td>
<td>lactoferrin, transferrin [121]</td>
</tr>
<tr>
<td><em>Streptococcus pneumoniae</em></td>
<td>Pneumonia, meningitis, bacteremia, oral media</td>
<td>lactoferrin, transferrin [121]</td>
</tr>
<tr>
<td><em>Legionnaire</em></td>
<td>Legionnaire</td>
<td>lactoferrin, transferrin [121]</td>
</tr>
<tr>
<td><em>Trompena pallidum</em></td>
<td>Sepsis</td>
<td>lactoferrin, transferrin [121]</td>
</tr>
<tr>
<td><em>Mycoplama pneumoniae</em></td>
<td>Pneumonia</td>
<td>lactoferrin, transferrin [121]</td>
</tr>
<tr>
<td>Bordetella pertussis</td>
<td>Whooping cough</td>
<td>lactoferrin, transferrin [121]</td>
</tr>
<tr>
<td><em>Chlamydia trachomatis</em></td>
<td>Nongonococcal urethritis, infertility</td>
<td>lactoferrin, transferrin [121]</td>
</tr>
<tr>
<td><em>Helicobacter pylori</em></td>
<td>Gastritis, gastric and duodenal ulcers, gastric adenocarcinoma, lymphoma</td>
<td>lactoferrin, transferrin [121]</td>
</tr>
</tbody>
</table>

possible that endosomes in cancer cells are less effective in transporting released iron out to the cytoplasm. One possible drawback of artemisinin-tagged transferrin is that it has to compete with endogenous transferrin for binding to the transferrin receptor [11]. A possible method to overcome this is to increase the tigogen efficiency such that more artemisinin molecules would be attached to a mole of transferrin. This has to be done such that the tigogen head does not effect the affinity of the transferrin to its receptor. By manipulating the transferrin-receptor iron-transport system of cancer cells, the efficiency of artemisinin and artemisinin-tagged transferrin can be enhanced. As transferrin receptors on the cell surface are coupled to other membrane components, means can be applied to modify the rate of iron delivery into cells. For example, the K562 cell line has been widely used as an in vitro model for CML. Various compounds have been shown to enhance iron content in K562 cells [12]. K562 cells have been shown to upregulate surface transferrin receptors, thus enhancing iron uptake in K562 cells [18]. Erythropoietin also increases transferrin receptor expression and intracellular iron content in K562 cells [18]. Thapsigargin has been shown to speed calcium-dependent iron uptake and increase the overall capacity of K562 cells in taking iron by accelerating the transferrin receptor endocytosis process [18]. Conceivably, these compounds could enhance the rate and specificity of artemisinin for the treatment of CML. Another interesting membrane component is the receptor for insulin-like growth factor I (IGF-I), which is endogenously tagged with transferrin receptors. IGF has been shown to enhance iron influx in cells, paradoxically, by reducing the rate of endocytosis, and allows more time for cell surface transferrin receptors to pick up transferrin. Furthermore, IGF-I receptors are expressed in higher amount in cancer cells than in normal cultured cells [12]. The rates of iron delivery to IGF-I and artemisinin-tagged transferrin could further enhance the potency and selectivity of the targeted-compound in killing cancer cells. Because artemisin and its derivatives are a free radical, its effectiveness could also be enhanced by increasing oxygen tension, decreasing intake of antioxidants, and blockade of prooxidase and catalase by drugs such as micareide, which will render the iron more available to be tigogenized so that they would not also increase the toxicity of artemisinin towards normal cells. Due to the emergence of multiple drug-resistant bacteria, there is an urgent need to develop new antibiotics [12]. Some of these drug-resistant bacteria, for example, *Staphylococcus aureus, Streptococcus pneumoniae, Pseudomonas aeruginosa, and Mycobacterium tuberculosis* are major problems of human health, particularly in developing countries. Because iron is an essential nutrient for growth, development and cellular functions, living organisms have evolved sophisticated mechanisms to obtain iron from the environment [11-13]. A strategy used by some bacteria is to hijack iron from iron-containing compounds endogenous to their hosts. For example, certain bacteria (see Table 2) have receptors on the surface to bind host transferrin and lactoferrin (an iron-carrying protein that is abundant in body fluids such as mucus). After binding, iron is released and actively transported into a bacterium [12]. These bacterial iron assimilation mechanisms can provide a basis for effective drug targeting and delivery.

Studies have shown that, in general, a high concentration of artemisinin is required to kill bacteria [12]. It is probably because iron is very tightly regulated at the bacteria. Artemisin is ineffective without iron. The question is whether or not artemisinin-tagged transferrin and lactoferrin are effective in killing bacteria that utilize these iron-transport proteins. The undetermined preliminary data have shown that artemisin-tagged transferrin is quite effective in inhibiting the growth of *M. tuberculosis* compared with isosorbid. *M. tuberculosis* has been shown to use transferrin and lactoferrin and are not very sensitive to iron [12]. Artemisin could also be tagged to other physiological iron-carrying molecules. For example, bacteria have two basic mechanisms to pick up iron from the environment. The first is to hijack iron from iron-containing compounds endogenous to their hosts, such as transferrin and lactoferrin, as discussed above. Another mechanism is secretion and retrieval of highly specific iron-binding chemicals known as siderophores. Two major forms of bacterial siderophores are carboxylate and hydroxamate that have very high iron affinity. They are synthesised and secreted by certain bacteria to scavenge iron from the environment. Bacteria have developed specific receptors to bind and pick up siderophores and transport them inside through molecular channels. Once inside, iron is released from the siderophores. Examples of bacteria utilizing the siderophore mechanism are *Escherichia coli, Salmonella typhimurium, Klebsiella pneumoniae* and *Pseudomonas*. There are also bacteria that use both host iron-carrying proteins and siderophores. Examples are *E. coli* and *M. tuberculosis*. Campylobacter jejuni and *Campylobacter jejuni* that do not produce siderophores, but pick up siderophores released by other bacteria. By the same token as the artemisinin-tagged transferrin, artemisin can be tagged to siderophores and these compounds may have potential for treatment of bacterial infection. Targeting artemisin to siderophores is relatively easy. Because the molecules are small, they can be administered orally. Also, as mammalian cells do not bind or retain potent forms of iron, they should be non-toxic to humans. Miller and co-workers have explored the receptor-mediated transport of siderophores to deliver antibacterial agents, by targeting *E. jejuni*, *S. aureus*, and other antibiotic compounds to siderophores [15-16]. These compounds kill bacteria at mid-to-high micromolar concentrations. Thus, the use of artemisinin-tagged siderophores for treatment of bacterial infection is feasible. Again, they provide the advantage and efficiency of bringing both artemisin and its targeting agent together simultaneously into a bacterium. It should also be pointed out that certain fungi also use siderophores to obtain iron [15]. Artemisin-tagged siderophores may therefore, also be useful for the treatment of fungal infection.

In general, compounds that contain iron-sensitive endoperoxide and physiological iron-carrying compounds tagged with endoperoxide, fit into the scheme of targeted therapy discussed in this paper.
Targeted treatment of cancer with artemisinin and artemisinin-tagged iron-carrying compounds

35. Table 1. Cancer cell in vitro growth inhibition activity and selectivity of anticancer compounds and endoperoxides.

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC50 (µM)</th>
<th>Selectivity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doxorubicin</td>
<td>0.17</td>
<td>NA</td>
<td>[88,90]</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>1.4</td>
<td>NA</td>
<td>[88]</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>0.01</td>
<td>NA</td>
<td>[88]</td>
</tr>
<tr>
<td>Docetaxel</td>
<td>0.5</td>
<td>NA</td>
<td>[88]</td>
</tr>
<tr>
<td>Troxerutine</td>
<td>0.04</td>
<td>NA</td>
<td>[88]</td>
</tr>
<tr>
<td>Vinorelbine</td>
<td>0.05</td>
<td>NA</td>
<td>[88]</td>
</tr>
<tr>
<td>Mitomycin C</td>
<td>3.5</td>
<td>NA</td>
<td>[88]</td>
</tr>
<tr>
<td>Etoposide</td>
<td>0.02</td>
<td>NA</td>
<td>[88]</td>
</tr>
<tr>
<td>Staurosporine</td>
<td>0.05</td>
<td>NA</td>
<td>[88]</td>
</tr>
<tr>
<td>Dihydropyranolactone</td>
<td>2.5</td>
<td>NA</td>
<td>[88]</td>
</tr>
<tr>
<td>Dihydropyranolactone + Troxerutine</td>
<td>1.5</td>
<td>NA</td>
<td>[88]</td>
</tr>
<tr>
<td>Artemisinin + Hoferafer</td>
<td>0.02</td>
<td>NA</td>
<td>[88]</td>
</tr>
<tr>
<td>Artemisinin + Troxerutine</td>
<td>0.02</td>
<td>NA</td>
<td>[88]</td>
</tr>
<tr>
<td>Artemisinin + Staurosporine</td>
<td>0.03</td>
<td>NA</td>
<td>[88]</td>
</tr>
<tr>
<td>Artemisinin + Dihydropyranolactone</td>
<td>0.03</td>
<td>NA</td>
<td>[88]</td>
</tr>
<tr>
<td>Artemisinin + Etoposide</td>
<td>0.02</td>
<td>NA</td>
<td>[88]</td>
</tr>
<tr>
<td>Artemisinin + Vinorelbine</td>
<td>0.02</td>
<td>NA</td>
<td>[88]</td>
</tr>
<tr>
<td>Artemisinin + Dihydropyranolactone</td>
<td>0.02</td>
<td>NA</td>
<td>[88]</td>
</tr>
</tbody>
</table>
The 'tagged compound' was compared with that of doxycycline-resistant. The following is the 
Cmax values of the 'tagged-compound' and DHA on Multi-4 cells and lymphocytes, as determined by 
the Probit analysis: Multi-4-compounded 0.98 μM, Multi-4-DHA 1.6 μM, and Multi-4-compounded 
33 μM, lymphocyte-0.14, 58.4 μM. Thus, compared with 
DHA, the 'tagged-compound' is more potent in killing Multi-4 cells and less potent in killing normal lymphocytes.

DHA is inducing more killing in Multi-4 cells than its normal counterpart, whereas for the 'tagged-compound', it is 34 times higher.

6. Expert opinion

Anticancer is an effective anticancer compound that produces 
side effects. Because it is inexpensive and effective, orally, it could be a useful anticancer agent for patients in 
developing countries. However, it is not suitable for people 
with severe kidney or liver problems. We have observed 
that the coadministration of anticancer and 
pharmacokinetics in humans. Some may be more effective 
against certain types of cancer than others. For example, 
the glycoalkaloid analog is more hydrophilic and readily 
searches the blood-brain barrier. It may be more 
selective for the treatment of brain cancer. The highest 
concentration of anticancer was found to be in the intravenous administration in the rat. If this is also true 
in humans, anticancer may be a better analogue for the 
treatment of colon cancer. Anticancer-tagged transfer is more selective than anticancer, but it is more expensive to 
perform. However, further technological developments, such as recombinant technology, may possibly lower the cost 
of this compound. Further studies are needed to study the mechanism of action and to confirm its effectiveness in 
patients with renal or hepatic impairment.

The transferring complex has more selective.

Further advantages of using anticancer for cancer treat-
ment are that it is effective orally and is non-toxic. In addi-
tion to extraction from the Artemisia plant, a technology 
that is being developed to genetically engineer bacteria to synthesize 
anticancer precursor, which would then be chemically 
converted to anticancer.

Anticancer-tagged transfer provides a new strategy for 
selecting anticancer compounds. In this case, anticancer is selectively 
brought into cancer cells, and release of iron from the trans-
ferin molecule indirectly triggers the cytokine action. 
Targeting the transferin to transferin also increases the half-life of anticancer in the body (17). The short half-life of 
anticancer and its analogs is a problem in its therapeutic 
effectiveness, because transferin is a natural compound and iron is an important compo-
nent in cellular function, it is less likely that tolerance would 
be developed to anticancer tagged transferin. However, the antitumoral effectiveness of anticancer tagged transferin in vivo remains to be studied.

5. Conclusion

A major consideration in effective drug therapy is selectivity as 
less drugs would be needed and less side 
effects would be produced. Artemisia fulfills this criterion by 
taking advantage of a major physiological difference between 
cancer and normal cells, the former 
upregulates more iron. Because this is true for most cancer cells, 
anticancer is conceivably effective against many types of 
cancer. A comparison of anticancer-like compounds and 
other drug therapy systems is shown in Table 1. In 
general, the in vivo effective doses of anticancer-like 
compounds are comparable to those of traditional 
chemotherapeutic agents. However, the anticancer 
compounds have much higher selectivity.

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Targeted treatment of cancer with artemisinin and artemisinin-tagged iron—driving compounds

Hydrophobic interaction high performance liquid chromatography (HPLC) was used to determine the purity of the tagged prodrug. Because artemisinin is a hydrophilic compound, artemisinin-tagged protein moieties show lower HPLC conditions. The peak of tagged transferrin was much broader than native transferrin, suggesting that the authors' sample was a mixture of tagged prodrugs with different number of artemisinic acid moiety on the protein surface. Matrix-assisted laser desorption/ionization mass spectrometry confirmed the HPLC data. Tagged transferrin and native transferrin gave their molecular ion peaks at 77,619 Da and 75,828 Da, respectively. The mass difference corresponded to the tagging of 4.2 artemisinic moieties per protein molecule. The peak shape of tagged prodrug was again, much broader than that of native transferrin.

The authors were able to show that artemisinin moieties in tagged prodrug contain the intact endopeptidase bond. Both DHA and artemisinin produce chemiluminescence when mixed with hematin and luminol due to their endopeptidase bond. When tagged protein was reacted with the chemiluminescent reactant (hematin and luminol), the solution produced a time-dependent chemiluminescence signal similar to that of dihydroartemisinin.
transfers transferrin and the iron it carries into cells. Because cancer cells require a large amount of iron, they express a high number of transferrin receptors on their surface and uptake more iron during G1 and S phases of the cell cycle. The authors speculate that if transferrin is covidentally attached to hologranin, it would be transported as a pack- age into cells and react with iron within the endosome where iron is released from hologranin. This may enhance the cytosolic 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 (7,15). Transferrin has two N-glycoseides attached to the N-terminal domain (16). Periodate oxidation of these carbohydrate chains generates reactive aldehyde groups that can be modified with a variety of hydra- zine or aminohydrazone derivatives of artemisinin. Thus, the artemisinin analogue artemolic acid has been tagged to the glycosyl-moiety of hologranin transferrin using a relatively simple process. The authors estimate that as many as 10 artemisinin derivatives could be tagged to a molecule of transferrin. The following is a brief description of the procedures that may one use to synthesise artemisinin-tagged transferrin.

4.1 Method of tagging artemisinin to transferrin

The two N-glycoseides attached to the apurinie residues in the C-terminal domain of a transferrin molecule are not involved in binding of transferrin to its receptor. Periodate oxidation of these carbohydrate chains generates reactive aldehyde groups that can react with hydrazone or aminohydrazone derivatives of artemisinin. The carbohydrate chains of transferrin is primarily a lanarre saccharide (Figure 2). Assuming that all 1,2-diol moieties are oxidised to the corresponding aldehyde group, the authors estimate that >10 artemisinin units could be tagged to a molecule of hologranin transferrin. Artemisinin has also been tagged to amino acid side chains, such as lysine, on the protein surface. Such lysine-based tagging strategy could, however, interfere with receptor binding. The authors have shown that carbonyl-centric tagging chemistry produced a more potent tagged protein compared to lysine-based tagging chemistry (34).

Arteolic acid was used for the tagging. Arteolic acid has a rigid spacer to keep the bulky artemisinin moiety away from the carbohydrate array. Arteolic acid methyl ester was first synthesised (35), and then reacted with hydrazine to produce the desired arteolic acid hydrazine (ART-Ph-NH₂) (Figure 3). Hologranin transferrin was then oxidised by sodium periodate in acetate buffer, pH 5.5, for 20 min. Excess reagents and by-products were removed by gel filtration chromatography on Sephadex G-25. The protein fractions were combined, and immediately reacted with arteolic acid hydrazine to produce the tagged transferrin (Figure 4). The product was purified by the second gel filtration column (Sephadex G-25) that had been equilibrated with saline buffer, pH 7.5. The protein fractions were collected and stored at 4°C. Unlike oxidised transferrin, tagged protein was found to be very stable, and can be stored at 4°C for as least 12 months without any significant change in its biological activities.

Ultraviolet, visual and circular dichromism spectra of artemisinin-tagged transferrin showed that the protein structure remained largely intact during the protein modification. However, the iron content decreased from ~100 to 40% during the periodate oxidation step. The iron content remained unchanged during the tagging step. Periodate, therefore, damages the iron binding site, possibly by oxidising some coordinating amino acid residues such as Tyr and His. Procedures to prevent this damage are now being developed in the authors' laboratory.