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Expanding the Therapeutic Spectrum of Artemisinin: Activity Against Infectious Diseases Beyond Malaria and Novel Pharmaceutical Developments

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ABSTRACT

The interest of Western medicine in Traditional Chinese Medicine (TCM) as a source of drug leads/new drugs to treat diseases without available efficient therapies has been dramatically augmented in the last decades by the extensive work and the outstanding findings achieved within this kind of medicine. The practice of TCM over thousands of years has equipped scientists with substantial experience with hundreds of plants that led to the discovery of artemisinin (*qinghaosu*), which is extracted from the medicinal plant *Artemisia annua* L. (*qinghao*). The unexpected success of artemisinin in combating malaria has drawn strong attention from the scientific community towards TCM. Artemisinin was discovered by Youyou Tu in 1972. Since then, several novel pharmacological activities based on the well-known properties of the sesquiterpene lactone structure with the oxepane ring and an endoperoxide bridge have been unravelled. Beyond malaria, artemisinin and its derivatives (artemisinins) exert profound activities towards other protozoans (*Leishmania*, *Trypanosoma*, amoebas, *Neospora caninum*, and *Eimeria tenella*), trematodes (*Schistosoma*, liver flukes), and viruses (human cytomegalovirus, hepatitis B and C viruses). Less clear is the effect against bacteria and fungi. Based on the promising results of artemisinin and the first generation derivatives (artesunate, artemether, arteether), novel drug development strategies have been pursued. These included the synthesis of acetal- and non-acetal-type artemisinin dimeric molecules as well as developing nanotechnological approaches, *e.g.* artemisinin-based liposomes, niosomes, micelles, solid lipid nanocarriers, nanostructured lipid carriers, nanoparticles, fullerenes and nanotubes. The current review presents an overview on different aspects of artemisinins, including sources, chemistry, biological/pharmacological properties, types of infectious pathogens that are susceptible to artemisinins *in vitro* and *in vivo*, in addition to the advancement in their drug delivery systems utilizing pharmaceutical technology. It would be expected that different therapeutic strategies based on the second and third generation artemisinin derivatives and artemisinin-based drug technologies would be available in the near future to treat specific infectious diseases.

Key words: Artemisinin derivatives, *Artemisia annua*, Asteraceae, Antimalarial drugs, Anti-pathogen activity, Antiviral properties, Artemisinin-loaded nanocarriers, Traditional Chinese Medicine

Abbreviations: ARM: Artemether; ARM-LNP: Artemether-loaded lipid nanoparticles; ART: Artemisinin; ACT: Artemisinin-based combination therapies; AC-PL: Artemisinin-curcumin-loaded PEGylated liposomes; ADPs: Artemisinin dimer piperazine derivatives; A-CL: Artemisinin-loaded conventional liposomes, artemisinin-curcumin-loaded; AST: Artesunate; BBB: Blood-brain barrier; AC-CL: Conventional liposomes; A-PL: artemisinin-loaded PEGylated liposomes; DHA: Dihydroartemisinin; %EE: Entrapment efficiency; GNO: Ground nut oil; kDNA: Kinetoplast; LNs: Lipid nanospheres; NPs: Liposomal nanoparticles; MRT: Mean residence times; MPEG: Methoxy polyethylene glycol; NP: Nanoparticle; NLC: Nanostructured lipid carrier; NCEs: New chemical entities; PSM: Plant secondary metabolites; SNL: Solid lipid nanovectors; TCM: Traditional Chinese Medicine; TR: Transferrin; TNBC: Triple negative breast cancer; VM: Vasculogenic mimicry

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INTRODUCTION

Since the beginning of 1950s, a tremendous effort has been undertaken to identify novel drugs from natural sources with a goal to develop efficacious treatments for different human diseases[1]. Nature has been a valuable source to reach this objective. Inspired by this objective, Western medicinal approach has been interested in active molecules occur in commonly used medications derived from traditional medicine. That has resulted in the re-discovery of highly-effective drugs such as camptothecin[2], paclitaxel or artemisinin among a long list of natural products[3-4].

According to an analysis by Newman and Cragg[5], 50% of all new chemical entities (NCEs) and 64% of all small-molecule NCEs of drugs approved by the US FDA between 1981 and 2010 were natural products, natural products derived compounds, or compounds inspired by natural products[5]. A particularly rich source of these compounds has been traditional Chinese medicine (TCM). The long experience with this discipline over millennia has led to the discovery of hundreds of remedies for the treatment of different diseases and ailments[6-9]. Chinese medicinal plants in particular have contributed a great deal by providing structurally diverse and biologically active compounds[10]. One of the most prominent examples is the discovery of artemisinin in the Chinese medicinal herb *A. annua* L. (黄花蒿, *huang hua hao*), which had also been in use in traditional European medicine. The genus *Artemisia* (Family Asteraceae) comprises more than 400 species which are distributed in the northern hemisphere of Eurasia and America[11] Many *Artemisia* species such as *A. abrotanum, A. absinthium, A. annua, A. apiacea, A. cina, A. dracunculoides, A. frigida, A. glacialis, A. herba-alba*, *A. maritima, A. mexicana, A. pontica, A. tilesii, A. tridentata,* and *A. vulgaris* have been and are still used in traditional medicine and in phytotherapy (reviewed in:[12-13]). Initially, it was assumed that artemisinin was isolated from the plant *qing hao* (*A. apiacea*), and that is the reason it was called *qinghaosu* (Chinese: 青蒿素). Later on, it became known that it is derived from *A. annua*, a plant that has been used in China for fever, headaches and malaria[14-15].

In 1967, China launched the 523 research program (named after its official starting date, 23rd of May), which was aimed at the discovery of new malaria treatments, since resistance to the well-established drugs had become a severe problem. A multidisciplinary team headed by Youyou Tu which included both phytochemical and pharmacological researchers was formed at the Institute of Chinese Materia Medica, China Academy of Chinese Medical Sciences, with the task of working on the extraction and isolation of constituents with antimalarial activities from Chinese herbs. The team investigated more than 2,000 Chinese herb preparations and identified 640 hits with antimalarial activities[16-17]. More than 380 extracts obtained from ~200 Chinese herbs were subjected to evaluation in a mouse model of malaria. However, *A. annua* extracts showed no reproducible results because of instability of artemisinin. The information that heat should be avoided during extraction, came from Ge Hong's Handbook of Prescriptions for Emergencies: "A handful of *qinghao* immersed with two liters of water, wring out the juice and drink it all". Since the *A. annua* extract appeared to be active, but also toxic to animals, it was separated into an acidic portion with no antimalarial activity and a neutral extract, which showed reduced toxicity, but improved antimalarial activity. During the Cultural Revolution, there were no facilities to perform human trials of new drugs. Therefore, the Chinese research team acted as the first group of volunteers and took the extract themselves. Later, they went to Hainan to verify the efficacy of the extract in patients infected with both *P. vivax* and *P. falciparum*[18]. The extract produced very encouraging results.

Subsequently the group tried to isolate the active constituents of the extract. In 1972, they identified a colorless, crystalline substance with a molecular weight of 282.33 g/mol as the active component of the extract, and named it *qinghaosu* (artemisinin). Its structure was established by spectroscopic analysis, chemical reactions, and x-ray diffraction, and was firstly published in 1977[15]. It is a sesquiterpene lactone with the relatively rare secocadinane backbone. The most interesting part of the molecule is the oxepane ring with a peroxide bridge, which is necessary for its activity. Artemisinin was the first non-alkaloid antimalarial compound[19-20].

Early pharmacological studies showed that artemisinin had a direct lethal effect on *Plasmodium* by interfering with its mitochondrial function[21]. Pharmacokinetic studies revealed that artemisinin absorption, distribution, metabolism, and excretion are fast. Youyou Tu reported the 'Chemical studies on *qinghaosu* as a keynote speech during the 4th Conference of Tropical Diseases and Chemotherapy for Malaria sponsored by UNDB, World Bank and WHO in Beijing in October 1981[22]. Subsequently, it was decided to develop artemisinin as a new antimalarial drug.

In the following years, interest in artemisinin research expanded globally and was supported by WHO and World Bank[23-25]. Although artemisinin can be isolated relatively easily and with a yield of about 0.1% from *A. annua*[26], the synthesis was a goal. However, it turned out to be very challenging because of the many asymmetric carbon atoms in the molecule. Hence, the previous synthetic routes, some of which even led to the enantiopure product, were economically not competitive due to the complexity and the low yield[27-30]. Many scientists have tried to chemically modified artemisinin to improve its pharmacological characteristics. Semi-synthesis starting from artemisinin led to the development of chemically modified artemisinins with better solubility, absorption and efficacy, such as, dihydroartemisinin (DHA), a lactol, artesunate, a water-soluble succinate, which is more active and less toxic, and artemether a lipid soluble methyl-ether derivative, which is a lipid soluble form that has the longest half-life, but also the most toxicity, compared to arteether and artelinate. All these derivatives share an endoperoxide bridge in artemisinin and hence its pharmacological properties[31].

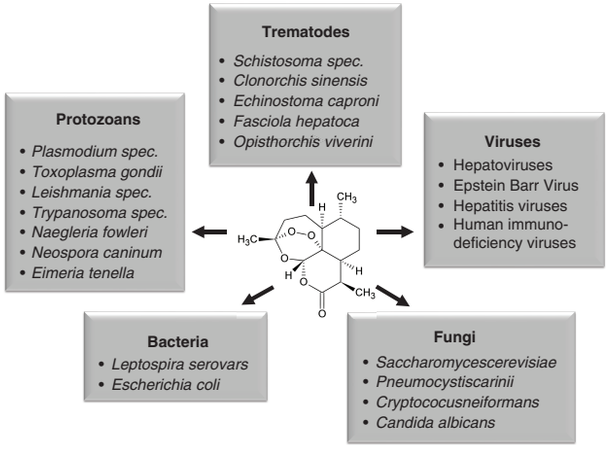


Figure 1: Overview of artemisinin's activity against infectious diseases.

The synthesis of these two derivatives needs an intermediate, DHA, which first was considered to be unstable. Later on, it was discovered that both artemether and artesunate were pro-drugs and had to be metabolized into DHA to become active in human blood. Thereafter, DHA was recognized as the active metabolite of all known artemisinin derivatives[32].

Via a publication of Welcome Trust, Rhône Poulenc Rorer (RPR, now Sanofi) became interested as first Western company in 1989 and decided to study the potential of these drugs and license one of them, the injectable artemether, from Kunming Pharmaceuticals[22]. Artemisinin and its semi-synthetic derivatives possess the most rapid action of all current drugs against *Plasmodium falciparum* malaria, and therefore artemisinin was recommended by WHO in combination with other antimalarial drugs to treat drug-resistant *Plasmodium falciparum* strains, cerebral malaria and malaria in children[22].

Because of the importance of this drug, Youyou Tu was awarded the 2011 Lasker-DeBakey Clinical Medical Research Award for discovering artemisinin as a treatment for malaria[33-34], and finally, as the first Chinese scientist, with the 2015 Nobel Prize in Physiology or Medicine[35-36].

Beside the anti-parasitic activity, artemisinin and its semi-synthetic derivatives have been evaluated *in vitro* as antiviral and antitrypanosomal agents with promising results[23-27]. Moreover, other authors have attempted these artemisinin derivatives for the treatment of a long list of human, animal and plant infections, mainly these caused by trematodes and protozoans, in addition to bacterial and fungal infections (**Figure 1**).

The aim of this review is to present progress in different aspect of artemisinins, with emphasis on their pharmacological properties, their therapeutic usefulness in treatment of infections inflicted by different pathogens, and the advancements in their drug delivery systems. The first part of this review provides an overview of the potential of artemisinin and derivatives to treat infectious diseases. The second part will focus on the development of nanocarriers loaded with artemisinin and its derivatives.

Activity Towards Protozoans

Plasmodium falciparum

Since the anti-parasite activity of artemisinin against *Plasmodium* was first discovered almost half a century ago (see above; for recent reviews[37-38], a large number of studies have been carried out, from early preliminary results to sophisticated meta-analyses. However, there have been still several issues on the artemisinin-based treatments that need to be clarified.

Treatment regimen approved by WHO

Recently the approval by the European Medicines Agency of a vaccine against malaria has opened a new approach to protect the population against exposure to parasite infection[39]. However it is estimated by WHO that in 2015, 214 million people worldwide suffer from malaria, the statistics confirm at least two millions of fatalities per year due to this disease[40]. The available anti-malaria drugs have become ineffective in all the cases due to the emergence of drug resistance and severe adverse effects. To treat the resistant strains of *Plasmodium*, WHO recommended artemisinin-based combination therapies (ACT)[41]. Artemisinin-based regimes are active for only 48 h to 72 h. However, this time is enough to dramatically reduce the number of parasites that are generated by asexual cycle as well as the production of gametocytes. The latter effect is very important to inhibit the transmission of the parasite[42-46].

How artemisinin and its derivatives act against the parasite

After its replication in the host liver, *Plasmodium* invades red blood cells, where to survive it degrades hemoglobin and consumes the liberated amino acids. When treated with artemisinins the release of heme-iron into the feeding-vacuoles of the parasite breaks the endoperoxide bridge of artemisinins by a Fenton reaction[47-50]. This cleavage produces reactive oxygen species that cause lethal effects on the *Plasmodium* vacuoles, resulting in the autodigestion of the parasite. The radicals produced in the Fenton reaction are not only the classical hydroxyl and superoxide anions, but also carbon-centered radicals, which are able to alkylate intracellular proteins in the parasite, such as the translationally controlled tumour protein (TCTP), a key-protein in the cell-cycle of the parasite[51-52].

Available analyses

The currently used drugs against malaria are amodiaquine, chloroquine, mefloquine, quinine and sulfadoxine-pyrimethamine, which are insufficient to efficiently combat the disease, because *P. falciparum* and *P. vivax* quickly develop resistance to these drugs and because many strains have become resistant. Artemether plus lumefantrine is the most recommendable combined regime to treat malaria caused by *Plasmodium*-resistant strains, not only because of its antimalarial effect, but also because it is blistered in one single oral preparation. An analysis with 1869 patients has demonstrated the success of this artemisinin-based combination to

overcome malaria resistance over other anti-parasitic treatments. Whether this regime is accompanied by gastrointestinal and/or neurological side effects is unknown, since clinical manifestation of the infection would mask any of these effects[53]. In another analysis with 4472 children, the failure of the treatment with artemether and lumefantrine was lower than that for amodiaquine plus sulfadoxine/pyrimethamine. However, the artemether and lumefantrine combination was more effective than artesunate plus sulfadoxine/pyrimethamine[54]. In another study, the artemether/lumefantrine combination had similar effects to those found in the DHA-napthoquine-trimethoprim regimen. These combinations seem to be better antimalarial therapies than amodiaquine plus artesunate, but worse than mefloquine and artesunate[55]. Recently a study comparing ACTs with alternative antimalarial regimens for treating acute uncomplicated *P. vivax* malaria concluded that in areas where chloroquine no longer cures the infection, DHA-piperaquine regimen may provide a longer period of post-treatment prophylaxis than artemether-lumefantrine or artesunate plus amodiaquine[56-57]. In spite of the successful results of artesunate-based monotherapy[53,55,58-60], WHO disapproved the treatment of patients in monotherapy with these artemisinin-derivatives[41].

Adverse effects

Artemisinin and its derivatives are safe compounds with a high tolerability. Because of mild side effects of these drugs, their combined regimes are useful for the treatment of children[53,59,61-66] and pregnant women[67-68]. Although adverse effects such anorexia, vomiting, nausea and dizziness are commonly observed[69], reversible neutropenia[70] or first-degree cardiac insufficiency are infrequent. Artemether as well as artemisinin cross the blood-brain barrier and some neuronal toxicity has been observed in *in vivo* models[71-73]. However, if artemisinins were tested in monkeys, it did not show any toxicity up to 292 mg/kg for a period of one to three months. This is equivalent to a dose of 20 g for a 70 kg human male subject[74]. The center of disease control (CDC) reported one single case of hepatitis due to artemisinin intake, but further investigations need to confirm this liver-specific toxicity[75].

Activity Towards Other Protozoans

Toxoplasma gondii

This protozoan infects approximately one third of the world population and is able to cause health problems varying from mild diseases in adults to death in foetuses and immunocompromised patients[76-77]. The treatment of *T. gondii* with spiramycine is effective in pregnant women, if the parasite has not crossed the placental barrier, but side effects are usually severe[78]. Thus, there is a need of finding safer drugs, such as artemisinin and artesunate, whose safety profile in previously treated pregnant women with malaria was high[67]. In the 1980s, Chinese scientists studied the effects of artemisinin and its derivatives against *T. gondii*[79]. These compounds completely eliminated the parasite from fibroblasts without toxic effects on cells. Arteether, sec-butyl-ether-artemisinin and in particular artemether were more effective than artemisinin[80]. Moreover, the new monoacetal derivatives of artemisinin have shown less toxicity and more activity *versus* this apicomplex parasite than trimethoprim-sulfamethoxazole (a mixture of antibiotics used against several bacterial infections) at an ID_{50} of 0.6 μg/ml (the same level as artemether, artemisinin and arteether[81]. However, *in vivo* models have shown controversial results to treat toxoplasmosis with artemisinin derivatives. Some studies obtained positive results with artemisinin and artesunate, but not with artemether against *T. gondii* in rats[82]. However, others found that artemisinin and artesunate are ineffective in rats, whereas new derivatives such us artemiside and artemisone were able to inhibit the cell cycle of the parasite *in vitro* and to increase the survival of infected mice[83] or that the thiazole CPH4-136, a new artemisinin derivative has showed moderate efficacy against *T. gondii* in *in vivo* models of acute and chronic infection in mice[57]. The mechanisms accounting for artemisinin activity against *Toxoplasma* has been widely investigated. Artemisinin derivatives inhibit several steps of *T. gondii* lytic cycle, such as tachyzoite replication, attachment and invasion of host cells[84]. Several studies highlighted the importance of intracellular calcium for *Toxoplasma* replication[85-86]. In fact, artemisinin inhibits SERCA (sarcoplasmic-endoplasmic reticulum calcium-ATPase) leading to changes in calcium homeostasis and therefore impairing key processes in the parasite physiology such as protein secretion, motility, infectivity and secretion[87-88]. In addition, peroxiredoxine is a cytoplasmic parasite protein, which is involved in antioxidant protection of the cell from radicals generated by artemisinin[89].

Leishmania

This protozoan infects its host via hematophageous mosquitoes, causing cutaneous and mucosa infections and, in some cases, such as the infection due to the genus *Donovani* (*Donovani infantum* and *archibaldi*) with visceral consequences that can be lethal for the host[90-91]. WHO estimates that there are 12 million people with undetected *Leishmania* infection and resistance to conventional treatment with pentavalent antimony and other drugs, such as amphotericine B and miltefosine. Leishmaniosis is a serious world-wide health problem specially in India and South-Asia[92]. An amino derivative of fluoro-artemisinin named BB 201 has demonstrated its anti-promastigote properties *in vitro* with IC_{50} of 1 μM, even in strains resistant to miltefosine and sitamaquine[93]. However, this study failed to find any effect of BB 201 on amastigote forms of *Leishmania*[93]. However, in another study artemisinin was found to be active against promastigote and amastigote forms with IC_{50} of 166 and 20 μM respectively, due to their ability to induce apoptosis in the parasite[94]. *In vitro* infection with *L. major* promastigotes was reduced after treatment with artemisinin with an IC_{50} of 0.75 μM. Moreover, artemisinin and artemether have demonstrated leishmanicidal activity against mastigotes in murine macrophages with an IC_{50} of 30 μM and 3 μM,

respectively[95]. Recently a study comparing ACTs with alternative antimalarial regimens for treating acute uncomplicated P. vivax malaria concluded that in areas where chloroquine no longer cures the infection, DHA-piperaquine regimen may provide a longer period of post-treatment prophylaxis than artemether-lumefantrine or artesunate plus amodiaquine[56-57]. C-9-artemisinin analogues are more active against *L. donovani,* if derivatization is carried out at the β-position than when α-position is used. It should be pointed out that the endoperoxide bridge of artemisinin-related compounds is also crucial for leishmanicidal activity[96-97].

Trypanosoma

These unicellular flagellate protozoa form a monophyletic clade within the class Kinetoplastida. Trypanosomes exist as parasites, which usually require more than one host. Many trypanosomes live in the blood of vertebrates, in which they are sometimes pathogenic. Blood-feeding insects, in which they live and multiply in the intestine, whereas stercorarian trypanosomes are transmitted via the faeces to the corresponding vertebrate host, generally transmit salivarian trypanosomes[98].

More than 25 species of *Trypanosoma* have been described. Of medical interest are *Trypanosoma brucei,* causing African sleeping sickness and *T. cruzi*, the pathogen of the American Chagas disease. Sleeping sickness in humans, which is geographically restricted to Africa is caused by *T. b. gambiense* and *T. b. rhodesiense*. The disease is abbreviated as HAT, human African trypanosomiasis. The closely related parasite *T. b. brucei* affects cattle, causing Nagana. Because of the close relatedness and lack of pathogenicity in humans *T. b. brucei* is often used in laboratory studies as a proxy for the human pathogens. HAT and Nagana are transmitted by tsetse flies of the genus *Glossina*. Vector control programs against tsetse flies were largely successful so that the number of infected patients is presently down to less than 17,000 individuals per year. But still, sleeping sickness is a very severe disease which lacks adequate chemotherapy[99-100].

The drugs used to treat sleeping sickness include pentamidine and suramin, which were developed more than 80 years ago. Newer drugs, such as melarsoprol and eflornithine came on the market 20-30 years ago. These drugs exhibit severe side effects and furthermore, several pathogens have become resistant to them[101]. Therefore, new drugs are urgently required. An alternative to synthetic drugs are plant secondary metabolites (PSM) which represent the active principles of medicinal plants[102]. Antitrypanosomal PSM include alkaloids, phenolics, saponins, steroidal glycosides, terpenoids (such as artemisinin) and polyacetylenes[100,101,103-105].

The synthetic drugs and PSM attack different molecular targets in trypanosomes[105]. Of these molecular targets, trypanothione is a unique antioxidant molecule in *Trypanosoma* and *Leishmania*[106]. It can be influenced directly (*e.g.* by antioxidants or polyacetylenes,[103,104]) or indirectly by blocking its synthesis via inhibition of ornithine decarboxylase (*e.g.* by eflornithine), alkaloids which can intercalate DNA (*e.g.* berberine and sanguinarine) are usually trypanocidal as they can interfere with the replication of kinetoplast (kDNA) and nuclear DNA[107-109]) and cause mutations. Another group of DNA-targeting drugs includes DNA topoisomerase inhibitors, such as camptothecin and dicentrine. Division of trypanosomes is also orchestrated by microtubules; thus microtubule inhibitors, such as vinblastine or colchicine also exhibit trypanotoxic effect[100,109]. A number of the aforementioned PSM induce programmed cell death (similar to apoptosis) in *T. b. brucei*[108].

It has been assumed that similarly to the effects observed in *Plasmodium*, artemisinin derivatives could generate bioactive radicals via its endoperoxide bridge that can attack trypanosome biomembranes. It can alkylate lipids, proteins and DNA in an iron-dependent manner and also depolarizes mitochondrial membranes[110,111]. Using a biotinylated probe of artemisinin with labeling affinity, Konziase (2015)[112] was able to detect a 60-kDA target protein for artemisinin in *T. b. brucei*, which might help to understand its molecular mode of action[110] in this parasite.

Typical IC_{50} values for artesunate against *Trypanosoma brucei* are close to 23 μg/ml[113], those of artemisinin around 36 μg/ml[114] as compared to IC_{50} values in the range of 0.9 to 19 μg/ml for synthetic drugs, such as diminazene, eflornithine, metronidazole, ornidazole and suramin. For *T. b. rhodesiense* the IC_{50} values for artemisinin were between 16 and 23 μM. The cytotoxicity was similar against *T. cruzi* and *Leishmania donovani*[111]. Artemisinin is thus less active against trypanosomes as compared to *Plasmodium*.

Methanol and dichloromethane extracts from *A. annua* are rich in artemisinin, artemisinic acid, arteanuine B and the coumarin scopoletin[113]. The dichloromethane extracts were more cytotoxic (IC_{50} values between 1.8 to 14.4 μg/ml than the methanol extracts (IC_{50} values from 10.8 to 77.5 μg/ml). The extracts were more potent than the isolated artemisinin, indicating that the components in the extract exhibit synergistic interactions, either by attacking different molecular targets or by enhancing the bioavailability of the sesquiterpenes[102].

IC_{50} values for dichloromethane extracts of *A. absinthium, A. abyssinica* and *A. afra* were in the range between 19 and 25 μg/ml[114]. Further *Artemisia* species with antitrypanosomal activity include *A. maciverae*[115], *A. maritima*[116], and *A. elegantissima*[117].

These findings suggest that artemisinin, its semi-synthetic derivatives represent interesting candidates as antitrypanosomal agents, whose specificity and activity need to be enhanced by chemical modification. But also the use of extracts should be considered as extracts consist of multicomponent mixtures, presumably, attacking different molecular targets, might help to prevent the development of resistant parasite strains[113].

Amoebiasis

Naegleria fowleri is a parasite that causes primary amoebic meningoencephalitis, a rare but fatal brain infection usually

acquired by via the nasal route. There is still no efficient therapy available. *In vitro* studies of the parasite revealed an active panel of drugs against *Naegleria*. However, no reliable therapy exists in clinical practice. Studies on the usefulness of artemisinin and its derivatives against this pathogen are few and controversial. Although *in vitro* studies have shown a good profile for artemisinin and its dihydro derivative[118], *in vivo* studies on treatment of amoebic meningoencephalitis were not very promising[119,120].

Neospora caninum

This apicomplexan parasite has a strong impact on farming industry, because the infection by this parasite is one of the most frequent causes of abortion in cattle. This was discovered late in 1988, due to misdiagnosis with *T. gondii*[121]. The parasite also infects dogs and perhaps humans, although this possibility has not completely been clarified yet. There have been more than 40 compounds tested against *Neospora,* but currently drugs are not available to treat the infection in cattle[122,123]. The reason is that drugs assayed for long-term treatment in bovine are uneconomic or produce metabolites that appear in milk or meat. However, prevention of *Neospora*-induced abortion is high interest. *In vitro* research with artemisinin has shown promising results. Artemisinin was able to eliminate *Neospora* tachyzoites from *in vitro* cultures at concentrations of 10-20 μg/ml within 11 days. On the other hand, low concentrations such as 1 μg/ml artemisinin had the same effect in a longer time period of 14 days. Even 0.1 μg/ml artemisinin were still active in reducing intracellular replication of this parasite[124]. Artemisone (10-amino-artemisinin derivative) was able to inhibiting *in vitro Neospora* cell cycle and to avoiding typical cerebral symptoms in a gerbil model of infection[125].

Eimeria tenella

This is the major cause of coccidian infection in chicken. Its main manifestation is bloody diarrhoea that leads to the animal death. As other apicomplex parasites that affect life stock and agricultural industry, the available drugs induce the development of resistance and/or their use in long-term treatments reduces the quality of the meat for human consumption. Even at low doses (1 to 2.5 mg/kg), artemisinin decreased the levels of oocysts of *Eimeria tenella, E. acervulina, E. maxima* and of a mixed infection of the three strains, in faeces of treated chickens[126-129].

Activity Towards Trematodes

Helminths are parasitic worms that represent a worldwide health problem. They are classified as cestodes, nematodes and trematodes, which are divided in two main families: blood flukes (*Schistosoma*) and liver flukes (*Clonorchis, Fasciola* and *Echinostoma*)[130].

Whereas semisynthetic artemisinins were not active against cestodes or nematodes such as *Gnathostoma spinigerum*[131] and *Paragonimus wertemani*[132], the antischistosomal activity of artemisinin-type drugs is remarkable.

Schistosoma

WHO calculates that more than 200 million people are affected with *Schistosoma haematobium, S. intercalatum*, *S. japonicum*, *S. mansoni* or *S. mekongi*[133-136], being sub-Saharan Africa geographical area with more people suffering from blood-fluke related diseases, from allergic reactions to death (200,000 deaths per year), which in most cases cannot be prevented by treatment with currently available drugs, such as praziquantel[136]. Artemisinin has once proven as a choice of therapy to treat schistosomiasis[137-138]. The same is true for DHA, which prevents worm development and histopathological damages in mice[139]. Artemether and artesunate have displayed anti-parasite activities in a dose-dependent manner in mice infected with *S. mansoni* and markedly reduced worm-burden[140]. Artemether is also active against 2-3 weeks old schistosomes and also juvenile *Schistosoma*, reducing morbidity due to the inhibition of egg production by the worm[141]. Combined therapy artemether with praziquantel seems to be the most effective treatment, because it is active towards all parasite life forms[142]. A new compound named DW-3-15 obtained by conjugation of artesunate and praziquantel has been tested in *S. japonicum* and may damage the tegument of male schistosomes[129].

As a monotherapy, artemether has been tested in randomized, double-blind placebo controlled trials in children against *S. mansoni*[143-144] and *S. haematobium*[144]. The results were encouraging, because patients treated with 6 mg/kg in six doses once every three (*S. mansoni*) or four (*S. haematobium*) weeks have encountered reduction in the incidence of infections. The dose profile was safe and with a high tolerability. In the study against *S. haematobium*, micro- and macrohematuria were decreased. Moreover, malaria parasitemia in cases of coinfection was inhibited at the same time[144].

Utzinger et al.[145] reported that artesunate prevents infections with *S. japonicum.* It could also be useful to treat schistosomiasis with an acceptable safety profile[146-148]. The combination of praziquantel and artesunate cured 81% of *S. japonicum* and *mansoni* infections[149]. Two studies in children in North Senegal showed that artesunate at concentrations recommended for malaria treatment was also effective against *S. haematobium*, reducing significantly the number of eggs in highly infected children without adverse side effects[62]. However, these results in *S. haematobium* and with other artemisin-derivatives are still controversial[150,151], especially if there is a coinfection with *Plasmodium*[152].

Liver flukes

Clonorchis sinensis is the third most prevalent liver worm parasite affecting humans in Asia as continent with the highest prevalence of this infection[153]. Artesunate and artemether reduced the parasite presence in rats until total

clearance, whereas standard drugs were not as effective in this animal model[154,155].

With regard to *Echinostoma caproni,* artemisinin-associated drugs have revealed a dose-dependent anti-parasitic activity *in vitro* and *in vivo*. Artemisinin showed a weak effect on the worm *in vitro* at 100 mg/ml for 72 h, whereas artesunate and artemether were effective at the same concentration, but for shorter periods of exposure (24 and 48 h, respectively). These derivatives have displayed dose-dependent effect *in vivo*[156].

Fasciola hepatica is another human liver fluke, which is worldwide distributed and which affects cattle and sheep[130,153]. The disease provoked by this parasite, fasciolosis, is mainly treated with triclabendazole, the most effective drug against *F. hepatica* as of yet. However, the drug usually induces drug resistance. Treatment with artemether of triclabendazol-resistant *F. hepatica* led to tegument and gut damages in the parasite[157] as well as had a severe impact on egg production[158]. In a rat model, another study showed the efficacy and safety of this artemisinin derivative on sheep by reducing egg and worm burden without adverse effect in adult animals. However, the possibility of embryotoxicity has been reported[159].

Opisthorchis viverrini is a liver fluke that mainly affect people in Southeast Asia, due to their practice of eating raw fish[160]. The treatment for this parasite is extremely effective with a single dose of praziquantiel, but there is a concern about the development of drug resistance. Another issue is that this parasite is associated with the development of cholangiocarcinoma[161]. In a hamster model, artesunate and artemether inhibited worm-burden infections. However, toxic effects at the tested dose (400 mg/kg) have been observed[162,163].

Activity Towards Viruses

In 1982 Chinese scientists were the first describing the antiviral properties of artemisinin[164].

Herpes viruses

The viruses belonging to this family are cytomegalovirus, herpes virus 1, 2, 6, 7, 8, Epstein-Barr and *Varicella zoster* virus. All of them are able to produce latent infections in their host that could be reactivated during processes of immunodeficiency. Regarding the treatment of infections by herpes viruses, artemisinin has shown poor, if any, activity towards this family of viruses. However, artesunate has high antiviral activity against all subtypes of herpes virus, Epstein-Barr, herpes simplex 1 and human herpes virus 6A[165,166].

Cytomegaloviruses

Human cytomegalovirus (HCMV), a β-herpes virus or herpes virus 5, infects mainly salivary glands, but may spread to other tissues, causing an enhancement in cell volume together with cytoskeletal damages. This virus may be potentially pathogenic in new-borns (congenital CMV, which may produce hearing loss, vision impairment, mental retardation or death)[167] and owing to immunodeficiency in patients with AIDS or recently transplant recipients, in whom renal, gastrointestinal, neurological and pulmonary complications have been described[168]. Current drugs to treat infections by cytomegaloviruses such as ganciclovir, cidofovir and foscarnet share the same target, viral DNA polymerase. Unfortunately, long-term treatment with these drugs frequently results in the development of viral refractoriness to all of them, which account for the dire need for new highly effective therapies[169]. Artesunate has shown a similar inhibitory effect on ganciclovir-resistant HCMV strains, such as AD169, and ganciclovir-sensitive parental HCMV[165-166]. Artesunate reduced the viremia in a stem cell transplant recipient[170]. The drug seems to interfere with some critical points in the cell cycle regulatory process, such as NF-κB and Sp1 targets, which are critical for the survival of the virus[166]. Similar to HCMV, artesunate is also active against RCMV. This effect is enhanced in the presence of iron[165].

Hepatitis viruses

Hepatitis B virus

This virus belongs to the genus *Orthohepadnaviridae* and, due to its hepatotropism, once it produces chronic host infection may cause cirrhosis and eventually hepatocarcinoma[171]. WHO estimates that in spite of availability of a safe vaccine there are 300 million people infected with Hepatitis B (HBV)[172]. The currently available drugs to treat chronic hepatitis B are pegylated interferon and nucleosides such as lamivudine, telbivudine, entecavir and adefovir, which are not effective in all cases, frequently due to required underdosing because of undesirable adverse effects[173] or to the emergence of mutant viruses that are drug-resistant[174-175].

Using permanently HBV infected HepG2 2.2.15 cells as an *in vitro* model, artemisinin and artesunate revealed anti-HBV properties[176]. Both drugs reduced HBsAg released from infected cells, in micromolar ranges to the culture medium. Moreover, additive effects of artesunate and lamivudine were found. In addition, the concentration at which artesunate started to be active against HBV was 10 μM, which was similar to that found for HCMV[166]. Interestingly, this concentration is close to drug doses found in plasma of malaria patients treated with artesunate[177]. This may be taken as a hint that clinically relevant artesunate doses may be reachable to treat HBV-infected patients. Other artemisinin derivatives, anhydrodihydroartemisinin and 10-(2'-butyloxy) dihydroartemisinin, have also demonstrated in this *in vitro* model to have stronger anti-HBV activity than artesunate[178].

Hepatitis C virus

Similar to HBV, hepatitis C virus (HCV) represents a serious disease burden with 130 million people infected worldwide. Moreover a safe vaccine has not yet been developed. HCV, a Hepadnaviridae, produces chronic infection in 50-85 % of the cases[179], which may evolve to cirrhosis and hepatocellular carcinoma in 20% of these patients[180]. Standardized treatment of chronic HCV with pegylated interferon and ribavirin lacks sufficient efficacy and tolerability. An important advance in this

field has been the recent discovery of the effective and relatively safe combinations of novel direct-acting anti-HCV drugs, such as ombitasvir and ledipasvir (NS5A inhibitors), sofosbuvir (NS5B polymerase inhibitor), paritaprevir (NS3/4A protease inhibitor), ritonavir (CYP3A inhibitor) and daclatasvir (NS5A replication complex inhibitor). Unfortunately, in spite of it is high cure rates, these combinations are not available for all HCV patients. Moreover, there is pending the serious problem of the emergence of strains resistant to currently available drugs and an enhanced risk of hepatocellular carcinoma development. These reasons still support the need of new treatment strategies to manage these patients[181]. In this respect, it is interesting to mention that the inclusion of artemisinin in these interferon-free anti-HCV formulations has not been explored yet. The effect of artemisinin as anti-HCV agent was recently studied using the replicon model. The results of this study permit to conclude that artemisinin is effective against this virus *in vitro* and its antiviral potency was enhanced in the presence of hemin without toxic effect on the host cells[182]. Until the development of replicon model in 2005, there was no robust system to support HCV replication *in vitro*[183-184]. Owing to this deficiency, surrogated models of HCV, such as bovine viral diarrhoea virus (BVDV), have been used for many years to investigate new drugs for the treatment of Flaviviruses[185], Artemisinin was effective against BVDV and its effect was additive in combination with interferon or ribavirin[186]. DHA, anhydrodihydroartemisinin, 10-dihydroartemisinyl acetate and 10-dihydroartemisinyl perfluoropropionate, have also demonstrated antiviral activity in this surrogated model of HCV, by inhibition of viral propagation and/or decreasing the release of BVDV-RNA to the medium[178].

Other viruses

Artesunate exerted antiviral *in vitro* activity *versus* HIV-1 in the nanomolar range, inhibiting partially the cell cycle of two strains of this virus (Ba-L and NL4-3)[166]. Although the anti-HIV activity of artemisinin and its derivatives has not been studied in humans, there are clinical evidence on the effect of artesunate on HIV in coinfection with *P. falciparum*[187]. This study stressed the importance of the immune system for the clearance of the parasite and also that artesunate in combination with other antimalarial drugs is effective, but reveals some risks attributed to the development of neutropenia.

Artesunate did not show any effects on influenza A virus[166]. In contrast, papilloma viruses may be treated with DHA, although this is restricted to local lesions. Artemisinin derivatives revealed less clear effects on the viral replicative cycle, but reduced the proliferation of tumor cells that have been transformed by papillomaviruses[188].

Activity Towards Bacteria, Yeast and Fungi

Less is known about the effects of artemisinin and its derivatives on infections caused by bacteria. *Leptospira serovars* has been recognized as co-infecting agent in malaria patients and artemisinin demonstrated certain activity against this spirochaete bacteria in these patients[189]. The effect of this compound and its derivatives was observed in a wide panel of bacteria *in vitro*. It was concluded that only anaerobic and gonococci species are susceptible to artemisinin-type drugs. It has to be stressed that diperoxy-artemisinin was active against all the anaerobic bacteria analyzed in this study[190].

Septicaemia or systemic inflammatory response to infection includes organ injury events that may be fatal[191-192]. Artemisinin has shown protective effects on *in vivo* models of sepsis using mice infected with living *E. coli*, dead *E. coli* and lipopolysaccharide[193]. Artemisinin also exerted synergistic effects together with other antibiotics such as ampicillin and its anti-sepsis effects could be due to cytokine release rather than antibacterial properties[194-195]. However, these results were not supported by another study, where no activity was found against Gram-negative bacteria, such as *E. coli* nor Gram-positive ones, such as *Staphylococcus aureus*[196].

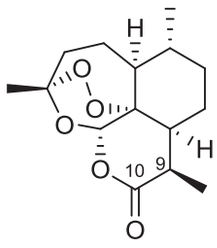
Despite the fact that *Saccharomyces cerevisiae* is not pathogenic yeast, several isogenic strains are useful to study molecular modes of action. DNA repair, checkpoint mechanisms and cell proliferation genes have been described to be pathways sensitive to artesunate[196-198]. These results may be taken as a hint that this drug acts by affecting mitochondria through ROS species, which interact with the electron transport chain.

The emergence of opportunistic infections is one of the main complications of acquired immune deficiency syndrome (AIDS)[199]. Several AIDS-related opportunistic pathogens without effective treatment options in AIDS patients are fungi, such as *Pneumocytis carinii* (or recently named *P. jirovecci*), *Penicillum marneffei*, and *Cryptoccocus neoformans*. Artemisinin was active against *P. carinii* without toxic effects in feeder cells[200]. In addition, DHA and artesunate were also effective in concentration ranges similar to those used for pentamidine[201]. Artemisinin also has presented fungistatic, but not fungicidal activity against the intracellular macrophage growth of *P. marneffei*[202]. Anhydrodihydroartemisinin reduced *C. neoformans* growth better than amphotericin B. The isomers α- and β-arteether also have a marked antifungicidal activity towards this parasite[203].

The effect of artemisinin-based therapies on *Candida* infections is still controversial. This fungus is the main cause of 90% of oral mycosis in immunosuppressed patients[204]. On the one hand, several extracts from *Artemisia annua* demonstrated strong antifungicidal activity against *C. albicans* and *C. glabrata*[196]. On the other hand, the effects of artemisinin or its derivatives against *C. albicans* have been described as being weak[203]. Moreover, Arteannuin B revealed inhibitory activity against *C. albicans* as well as some plants pathogens such as *Gaeunannomyces graminis var. tritici, Rhizoclonia cearealis*, *Gerlachia nivalis* and *Verticillium dahliae*.

Artemisinin Dimers as Activity-enhanced Derivatives

Artemisinin (**1**) and its monomeric derivatives have long been considered lethal substances for malaria parasite and hence a potent therapy for treatment of the mild and severe forms of malaria[205].



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However, the emergence of the parasite resistance against artemisinins, prompted scientists to develop artemisinin dimers, which have been proven to be far more powerful than the monomeric artemisinin counterparts[206-210].

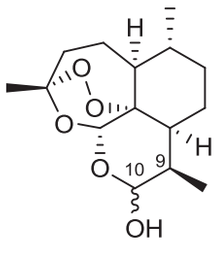
Synthesis and evaluation of artemisinin dimers for primarily utility of their antimalarial and anticancer effects have long been the focus of numerous research institutions worldwide. That has been driven by their outstanding inhibitory effect against the malaria parasite, in particular the chloroquine-resistant strains, and their anticancer potency. The bioactivities of these dimers are in most cases superior to the action of artemisinin, the monomeric derivatives and the standard drugs, sodium artesunate and doxorubicin, for malaria and cancer, respectively.

Artemisinin dimers were found to be thousand times more active than the monomers in the anti-cancer assays, in addition to exhibiting anti-leishmanial effect[211-214]. Furthermore, the dimeric analogues exhibit moderate anti-infective action, mainly against *Candida* species and *Cryptococcus neoformans*[214] and show inhibitory effect on HCMV replication[215-217].

Synthesis of artemisinin dimers

Acetal artemisinin dimers

Artemisinin dimers are divided into acetal and non-acetal types. The original dimers of artemisinin, prepared from DHA(**2**), were the acetal-type, without linkers, the symmetrical (α,α and β,β) and the unsymmetrical ones (**3**).



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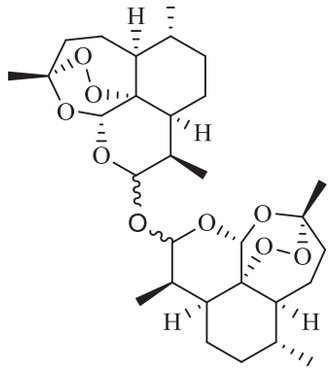

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These dimers were synthesized from the natural sesquiterpene peroxide artemisinin in two steps, the first involves reduction of artemisinin (**1**) to the hemiacetal DHA(**2**), with $NaBH_4$ in methanol at low temperature (1-5 °C) to avoid formation of by-products[218]. The second step is coupling two molecules of DHA using $BF_3.OEt_2$ as a catalyst[208,219]. The drawback of this reaction is that $BF_3.OEt_2$ is a non stereoselective catalyst and that results in the formation of a mixture of stereoisomers and large amount of unreacted DHA.

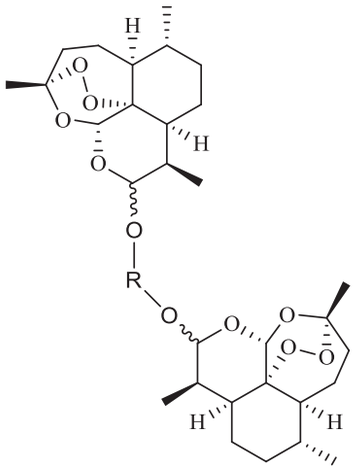
Later on, the synthesis of artemisinin dimers from DHA has developed to the introduction of different linkers between the two moieties of artemisinin (**4**). The nature of these linkers was either aliphatic chains of varying lengths, aromatic, heteroaromatic, or ethylene glycol or their homologs and bearing various functionalities. Synthesis of acetal-type dimers from DHA has been achieved using catalysts, such as $BF_3.OEt_2$, TMSC, $TMSOTf/AgClO_4$, and Grubbs catalyst. As a result of introducing these linkers, the anticancer and antimalarial activities of these dimers were dramatically enhanced.

The acetal dimers (**4**) are hydrolytically and metabolically unstable which is manifested by undergoing hydrolysis in acid medium and by the action of the ubiquitous esterases. That leads to liberation of DHA and loss of most of their activity rapidly.

Synthesis of C-10 carba trioxane dimers (non-acetal artemisinin dimers) (5)

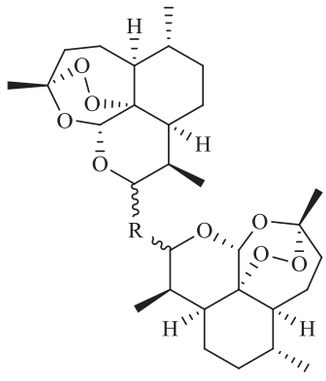
The instability of the acetal dimers has urged the chemists to find appropriate strategies to design and synthesize hydrolytically stable analogues[220]. In this respect, several synthetic approaches were pursued. One of these was substitution of the anomeric lactol hydroxyl group of DHA with fluorine, using diethylaminosulfur-trifluoride (DAST) followed by replacement of the fluorine with aryl, heteroaryl, and acetylid nucleophiles, using $BF_3.OEt_2$ as a catalyst to yield the C-10 non-acetal dimers[221-222]. Another strategy was based on conversion of artemisinin to the C-10 acetate followed by reacting the acetate with allyl bis-silane to afford a C-10 non-acetal dimer with a disubstituted olefin which was then functionalized to provide an array of derivatives[223].

A radically different approach utilizing artemisinic acid as a staring material, being cheaper than artemisinin was adopted by Jung et al[209]. In this method, artemisinic acid was converted to dihydroartemisinyl aldehyde by literature procedure, and then was reacted with vinyl-, 1-propenyl-, and –butenyl-magnesium chlorides to afford homologated alcohols. The second step was photooxygenative cyclization of the prepared olefinic alcohols, using O_2, Ros Bengal as photosensitizer, and tungsten light to yield artemisinin derivatives with alkenyl chains at C-10, where the C-O bond was replaced by a C-C bond. The alkenyl chain at C-10 of the artemisinin molecule was reactivated by transformation to alkyl bromide and was then catalytically coupled to another molecule of the C-10 alkyl bromide artemisinin, in the presence of a linker to yield the desirable dimers.



4, acetal artemisinin dimers

R=linker of any chemical nature



5, C-10 carba artemisinin dimers (non-acetal)

R=linker of any chemical nature

It is noteworthy that the non-acetal artemisinin dimers have been harnessed to prepare water soluble and orally administered derivatives for the pursuit of their biological properties *in vitro* and in animal investigations.

In vitro stability of acetal-type artemisinins versus the non-acetal ones

The non-acetal analogues were several times more stable in simulated stomach acid medium *in vitro* than their acetal counterparts[220]. DHA, arteether, and artelinic acid exhibited half-life of 17.19 h, 10.98 h, and 13.11 h, respectively, while closely related non-acetal derivatives, namely deoxoartemisinin, 10-(n-butyl)-deoxoartemisinin, and 10-benzyldeoxoartemisinin showed half-life of 213.36 h, 165.12 h, and 285.60 h, respectively. This experiment clearly indicated that converting the acetal to non-acetal (non-hydrolyzable) dimers is significantly enhancing the stability of these compounds, a highly desirable property of compounds to function as drug leads. The non-acetal or C-10-carba trioxane dimers (hydrolytically stable dimers) were found to be orally active[222-223].

In 2003, Posner synthesized and tested new C-10 carba trioxane dimers in *Plasmodium berghei*-infected mice. In combination with mefloquine, these dimers have been found to be more efficacious than the combination of artemether with mefloquine[224-225].

The concept of hybrid dimers was introduced to denote coupling artemisinin-derived core with a non-artemisinin molecule(s) attempting at enhancing the overall bioactivity of the compound. Representatives of this type of dimers are artemisinin-quinoline hybrid-dimers. They were synthesized by reacting DHA with series of aminoquinolines to yield acetal dimers with either one moiety of artemisinin and another of quinoline[226], or two moieties of artemisinin coupled to one molecule of quinoline[227-228]. The artemisinin-quinoline dimers were designed and synthesized on the premise that coupling a long acting antimalarial drug (an aminoquinoline residue) to artemisinin moiety will result in prolongation of the half-life of artemisinin. Experimentally, it did not extend the half-life of artemisinin. Besides, these dimers exhibited antimalarial effect which was less in magnitude than that of DHA. Nevertheless, they displayed anti- proliferating activity in tumor cell lines. On the stability side, they were not metabolically stable on exposure to esterases that resulted in hydrolysis and liberation of DHA with its undesirable properties, including rapid blood clearance and possible neurotoxicity[229]. Adding to this, they showed very low oral bioavailability, apparently owing to the high molecular mass of the molecules that comprise two moieties of artemisinin and one aminoquinoline residue[226].

Concerning the mechanism of action, it has been found in a number of acetal type dimers, as it happens in artemisinin molecule alone, that the effect is related largely to the generation of reactive oxygen species, attributed to the presence of the peroxide functionality, together with induction of apoptosis and inhibition of SERCA Ca^{2+} ATPase[212,230].

Nanotechnology to Increase Bioavailability and Efficacy

Formulation factors and technological processes can affect drug dissolution or release from the dosage form, drug absorption and stability (at the site of administration), or metabolic processes, resulting in the modification of bioavailability and bioequivalence of formulated drugs. Nano-sized drug delivery systems (10-400 nm diameter) holds huge interest in research because of their advantages in modifying pharmacokinetics and biodistribution, improving the therapeutic index of drugs by increasing their localization to specific tissues, organs, or cells, decreasing potential side effects, having an extreme versatility with regards to the route of administration[231].

The therapeutic value of artemisinins is limited due to a low bioavailability and a short half-life[232]. Promising novel nanoformulations loading artemisinins, such as liposomes,

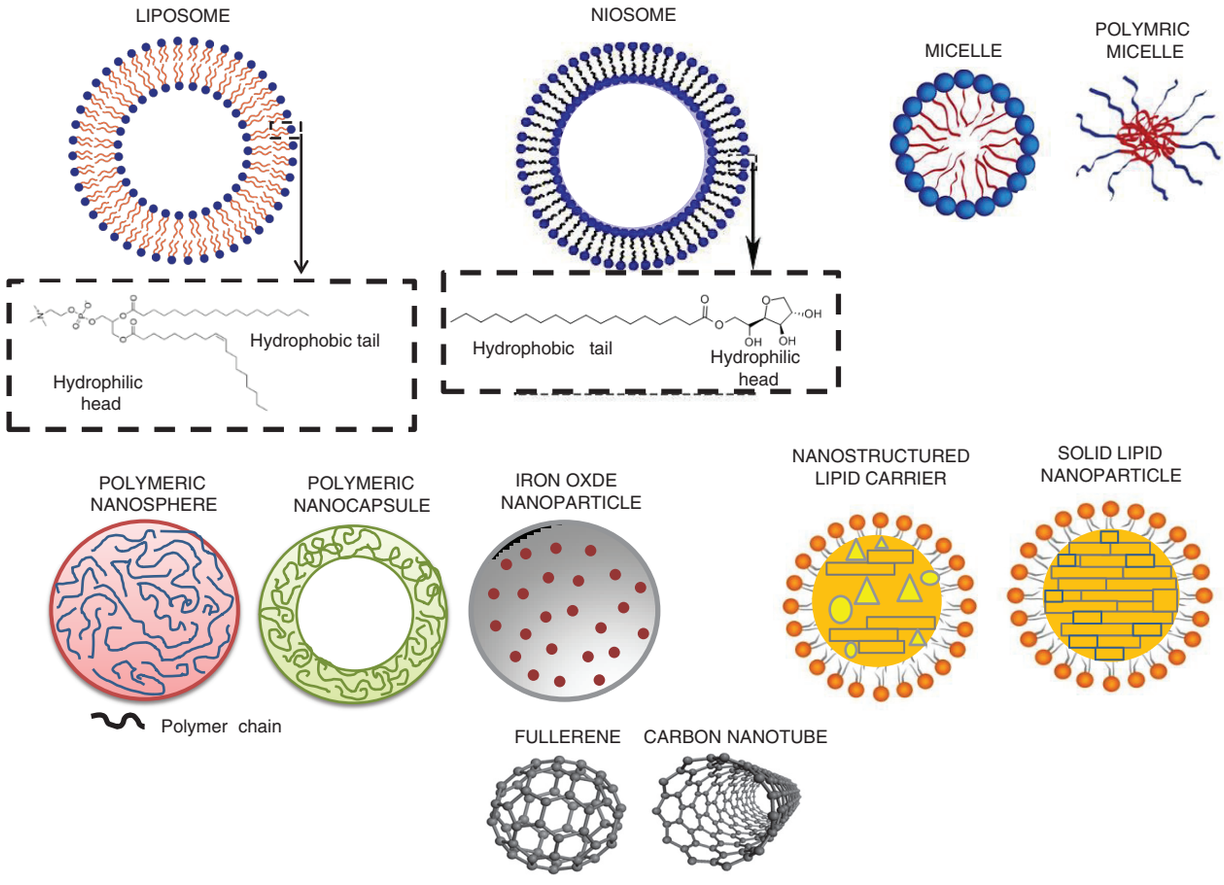


Figure 2: Overview of nanotechnological approaches based on artemisinin

micelles, solid lipid nanocarriers, niosomes, nanoparticles, fullerenes and nanotubes (**Figure 2**), offer significant promise in improving half-life, controlled release, better permeability, resistance to metabolic modification and highly specific site-targeted delivery of these therapeutic compounds. Remarkably, nanoparticulate drug delivery systems composed of biodegradable lipids or polymers, are biologically inert or weakly immunogenic, producing no antigenic or pyrogenic reactions, with a limited intrinsic toxicity.

Liposomes

Liposomes are spherical vesicles composed of one or more concentric lipid bilayers, separated by an aqueous medium. Lipids are phospholipids (synthetic or natural) plus cholesterol. They are versatile carriers designed to carry both hydrophilic substances (encapsulated in the aqueous compartment) and lipophilic molecules (inserted into the membrane).

Al-Angary and coworkers reported the first formulation based on liposomes in 1996[233]. Different liposomal formulations loaded with arteether were developed and their stability was evaluated. Additionally, the release of arteether from the liposomal systems was investigated. Generally, the increase of the acyl chain length as well as the addition of cholesterol caused a decrease in the arteether release rate.

Based on these studies, the same group of researchers tested *in vivo* (orally and intravenously administered) arteether loaded liposomes to New Zealand rabbits at a dose of 50 mg/kg[234]. Pharmacokinetic parameters after oral administration of liposomes were compared with those of oral aqueous suspension of micronized arteether. High bioavailability of arteether was evident in the case of liposome administration, with a faster rate and a better absorption of arteether with respect to those observed with aqueous suspension. Oral liposomes gave higher C_{max} and shorter T_{max} as well as a higher value of AUC. Almost complete arteether absorption was observed for oral liposomes with a relative bioavailability of 97.91% compared with the value of 31.83% found with the oral suspension.

Chimanuka and coworkers reported a further *in vivo* study with β-artemether liposomes[235]. Their therapeutic efficacy was tested in mice against *Plasmodium chabaudi* infection. A formulation containing 1.5 mg β-artemether (loading efficiency of ca. 100%, stable for 3 months at 4°C) was successfully used to circumvent the recrudescent parasitaemia[235].

Gabriëls and Plaizier-Vercammen evaluated the performance of liposomes containing artesunate, which has a rapid onset of therapeutic effect but suffers from a very quick elimination and as a consequence frequent administrations are required[236]. Liposomes loaded with 1 mg/ml artesunate

and encapsulation efficiency of ca. 100% were tested *in vitro* to evaluate the release of artesunate[236].

Liposomes based on artesunate were also evaluated for their cytotoxic activity against HepG2 cells. ICAA values of artesunate liposomes and artesunate were ca 16 and 20 μg/ml, respectively. Additionally, ICAA values of the same drugs against L-O2 normal human liver cells were ca. 100 and 106 μg/ml, respectively. Tumor growth inhibitory effect of artesunate nanoliposomes was 32.7%, while that of artesunate was 20.5%. HepG-2 cells treated with artesunate liposomes showed dose-dependent apoptosis. The antitumor effect of artesunate liposomes on human hepatoma HepG2 cells was stronger than that of artesunate at the same concentration[237].

Artemisinin-loaded conventional liposomes and polyethylene glycol (PEGylated) liposomes were also developed. Both liposomal formulations showed more than 70% of encapsulation efficacy with a mean diameter approximately of 130-140 nm and the polydispersity index of the formulations ranged from 0.2 to 0.3. Both formulations were suitable for intraperitoneal administration. Pharmacokinetic profile and the main pharmacokinetic parameters of the liposomes were evaluated in healthy mice. Free artemisinin was rapidly cleared from plasma and hardly detected 1 h after administration. Conversely, both liposomal formulations showed much longer blood-circulation time than free artemisinin; artemisinin was still detectable after 3 and 24 h of administration, respectively, for conventional and PEGylated liposomes, respectively. AUC (0-24 h) values were increased by approximately 6 times in both of the liposomal formulations, in comparison with free artemisinin. A strong effect of formulation on the half-life of artemisinin was enhanced by more than 5-fold by the incorporation of PEG into liposomes. Liposomes loaded with artemisinin, especially the long-circulating vesicles, could really represent a new strategy for developing smart, well-tolerated, and efficacious therapeutic nanocarriers to treat tumors, but could also be very useful to treat parasitic diseases[232].

The antimalarial efficacy of the developed novel liposomal delivery systems based on artemisinin or artemisinin-based combination therapy with curcumin has been investigated and reported in a further study. The *in vivo* activity was tested in *Plasmodium* berghei NK-65 infected mice, a suitable model for studying malaria because the infection presents structural, physiological and life cycle analogies with the human disease. Artemisinin, alone or in combination with curcumin, was encapsulated in conventional and PEGylated liposomes and the *in vivo* performance was assessed by comparison with the free drug. Mice were treated with artemisinin at the dosage of 50 mg/kg/days alone or plus curcumin as partner drug, administered at the dosage of 100 mg/kg/days. Non-formulated artemisinin began to decrease parasitemia levels only 7 days after the start of the treatment and it appeared to have a fluctuant trend in blood concentration, which is reflected in the antimalarial effectiveness. By contrast, treatments with artemisinin-loaded conventional liposomes (A-CL), artemisinin-curcumin-loaded conventional liposomes (AC-CL), artemisinin-loaded PEGylated liposomes (A-PL), artemisinin-curcumin-loaded PEGylated liposomes (AC-PL) appeared to have an immediate antimalarial effect. Both nanoencapsulated artemisinin and artemisinin plus curcumin formulations cured all malaria-infected mice within the same post-inoculation period. Additionally, all formulations showed less variability in artemisinin plasma concentrations, which suggested that A-CL, AC-CL, A-PL and AC-PL gave a modified release of drug(s) and, consequently, a constant antimalarial effect during time. In particular, A-PL seems to give the most pronounced and statistically significant therapeutic effect in this murine model of malaria. The enhanced permanency in blood of A-PL suggests the use of these nanosystems as suitable passive targeted carriers for parasitic infections. This strong effect of formulation is added up to the mechanism of action of artemisinin, which acts in the erythrocyte cycle stage of human host as a blood schizonticide[238].

Analogous PEGylated liposomes loaded with artemisinin, having a mean diameter of 455 nm, were tested against MCF-7 cell line culture. Encapsulation efficiency and the drug release were respectively ca. 92% and 5%. Cytotoxicity effect of the produced formulation was analysed by means of MTT method. PEGylated formulation had less IC_{50} compared to standard drug, i.e. 1.58 μg/ml *versus* 2.3 μg/ml[239].

Artemisinin dimers-piperazine conjugates were formulated in liposomes, which were efficiently released at acidic pHs that are known to exist within solid tumors and organelles such as endosomes and lysosomes. They down regulated the anti-apoptotic protein, survivin and cyclin D, when incubated at low concentrations with breast cancer cell lines. Liposome can also down regulate the oncogenic protein HER2, and its counterpart, HER3 in a HER2+ cell line. Furthermore, the wild type epidermal growth factor receptor (EGFR or HER1) declined in a triple negative breast cancer (TNBC) cell line in response to liposomes[240].

Conventional and PEGylated liposomes loaded with DHA have also been developed and tested for the cytotoxic effects against MCF-7 cell line. Both developed formulations show physical characteristics as drug carrier for parental administration and good values of encapsulation efficiency (71% conventional liposomes and 69% PEGylated liposomes). Physical and chemical stabilities were evaluated under storage condition and in presence of albumin. Cellular uptake efficiency of liposomes was determined by flow cytometry. Higher internalization occurred in the conventional liposomes rather than in the stealth liposomes suggesting that hydrophilic steric barrier of PEG molecules can reduce cellular uptake. Flow cytometry analysis was also used as an alternative technique for rapid size determination of liposomes. Cytotoxicity studies in the MCF-7 cell line confirmed the absence of toxicity in blank formulations suggesting liposomes may be a suitable carrier for delivery of DHA avoiding the use of organic solvents. Cytotoxicity of DHA and of both liposomal formulations was evaluated in the same cell line, confirming a modified release of DHA from vesicles after cellular uptake[241].

Recently, paclitaxel plus artemether liposomes functionalised with a mannose-vitamin E derivative conjugate (MAN-TPGS1000) and a dequalinium-lipid derivative conjugate (DQA-PEG2000-DSPE) were developed and tested on brain glioma cells in vitro and in brain glioma-bearing rats. The functional targeting was settled for transporting drugs across the blood-brain barrier (BBB), destroying vasculogenic mimicry (VM) channels, and eliminating cancer stem cells (CSCs) and cancer cells in the brain. The transport mechanism across the BBB was associated with receptor-mediated endocytosis by MAN-TPGS1000 conjugate via glucose transporters and adsorptive-mediated endocytosis by DQA-PEG2000-DSPE conjugate via electric charge-based interactions. The efficacy was related to the destruction of VM channels by regulating VM indicators, as well as the induction of apoptosis in brain cancer cells and CSCs by activating apoptotic enzymes and pro-apoptotic proteins and inhibiting anti-apoptotic proteins[242].

Recently, artemisinin and transferrin-loaded magnetic liposomes in thermosensitive and non-thermosensitive forms have been developed and evaluated for their antiproliferative activity against MCF-7 and MDA-MB-231 cells for better tumor-targeted therapy. The entrapment efficiencies of artemisinin, transferrin and magnetic iron oxide in the non-thermosensitive liposomes were ca. 89, 85 and 78%, respectively. Moreover, the thermosensitive formulation showed a suitable condition for thermal drug release at 42°C and exhibited high antiproliferative activity against MCF-7 and MDA-MB-231 cells in the presence of a magnetic field[243].

Lastly, artemisinin dimers-piperazine conjugates were formulated in liposomes based on 1,2-dipalmitoyl-sn-glycero-3-phosphocholine. Small unilamellar vesicles with an encapsulation efficiency greater than 90% and a size of ca. 80 nm were developed. Over 50% of the conjugates were released in 48 h at pH 4 compared with less than 20% at neutral. Liposomes exhibited high potency against human breast cancer and they were well tolerated well by non-tumorigenic cells. In MDA-MB-231 mouse xenograft model, liposomes were more effective than paclitaxel in controlling tumour growth. Cellular uptake studies showed endocytosis of the nanoparticles and release of core-trapped marker throughout the cytosol at 37°C[244].

Niosomes

Niosome is the acronym of "non-ionic surfactant-based" vesicle. They are structurally similar to liposomes but are prepared with non-ionic surfactant and cholesterol as ingredients, which make them more stable and thus offering many more advantages over liposomes.

Artemisone was encapsulated in niosomes prepared with sorbitan monostearate:cholesterol (3:1 ratio) and their effects against human melanoma A-375 cells and human keratinocytes HaCaT were evaluated. Encapsulation efficiencies was ca. 67% with average particle sizes of ca. 211 nm, while the zeta potential was −38 mV. The drug release after 7 hours was 85% of artemisone was released from the niosomes. The MTT assay indicates that formulation significantly suppresses melanoma cells ($P \leq 0.05$) in a dose-dependent manner. At 0.06 mg/mL, free artemisone suppresses almost 50% of the melanoma cell growth, whereas at this concentration niosome almost completely suppresses melanoma cell growth. Highly selective cytotoxicity towards the melanoma cells with negligible toxicity towards the normal skin cells was found and in the specific case of melanoma, as the nano-vesicles enhance skin permeation, artemisone loaded vesicles should be examined as a topical therapy for melanoma[245].

Micelles

Micelles are nanosized (typically in the range of 20–100 nm) supramolecular constructs formed from the self-assembly of amphiphilic molecules in aqueous environments. In water, the hydrophobic segment of the molecule self-associates into a semisolid core, with the hydrophilic segment forming a coronal layer. The resulting core-shell architecture is important for drug delivery purposes; the hydrophobic core serves as a reservoir for water-insoluble drugs.

Bilia and coworkers reported the solubilization of artemisinin by octanoyl-6-*O*-ascorbic acid (ASC8), a relatively novel surfactant that combines surface activity with powerful performance as radical scavenger[246]. Artemisinin was efficiently solubilized by ASC8 micelles, with no significant perturbation of the micellization.

Using DOSY NMR experiments the cmc of ASC8 (approximately 6 mM) was firstly evaluated. Artemisinin solubility in water was approximately 0.21 mM resulting in about 1 mM in the presence of 60 mM ASC8.

The same authors have investigated the solubilisation of artemisinin and curcumin, individually and in combination, in micelles of sodium dodecyl sulphate (SDS). The aqueous solubility of artemisinin was enhanced approximately 25-fold by 40 mM SDS, and 50-fold by 81 mM SDS, while that of curcumin was increased from 2 mM to 81 mM SDS. In addition, model studies on the use of the surface-active radical scavenger octanoyl-6-O-ascorbic acid (ASC8) to combine solubilisation with protection against oxidation for the chemically labile artemisinin were investigated. A 16-fold enhancement of artemisinin solubility was measured in a solution containing 40 mM SDS and 60 mM ASC8. Even after treatment with 60 mM hydrogen peroxide, more than a 30-fold excess, almost half the artemisinin remained, suggesting a potentially useful combination of the surface activity and antioxidant properties of the novel binary SDS: ASC8 system[247].

A new polymeric amphiphilic micellar system was developed for solubilization and controlled delivery of artemether. Methoxy polyethylene glycol (MPEG) 2000 and 5000 were used as hydrophilic terminal, which was linked to the hydrophobic di-fluorene methoxycarbonyl-L-lysine and to the two amino groups of l-lysine by consecutive peptide linkages and deprotection up to 2.5 generations. The half-generation (0.5 G, 1.5 G and 2.5 G) dendritic micelles of MPEG 2000 and 5000 were used to solubilize artemether. A considerable solubility enhancement of artemether up to three to fifteen times depending on concentration, generation

and type of dendritic micelles used was found. The dendritic carriers were proved to form stable micelles at 10-30 μg/ml depending on generation and type of MPEG used. The formulations increased the stability of the drug and also prolonged the release of artemether up to 1-2 days *in vitro*[248].

Solid lipid nanoparticles

Solid lipid nanoparticles (SLNs) are high stable nanocarriers (50-1,000 nm), having high protection against degradation of labile drugs and easily to produce on a large scale. They contain highly purified triglycerides, composed mainly of lipids that are solid at room temperature, which are stabilized by surfactants. Due to their small size and biocompatibility, SLNs may be used in the pharmaceutical field for various routes of administration, such as oral, parenteral, and percutaneous.

SLN made of Tween 80, Pluronic F68, and soya lecithin were loaded with arteether obtaining homogeneous particle size of ca. 100 nm and entrapment efficiency of ca. 69%. Pharmacokinetics studies indicated that their absorption has been significantly enhanced in comparison to arteether in aqueous suspension and arteether in groundnut oil in rats. The relative bioavailability of the SLN loaded with arteether to the arteether in groundnut oil and arteether in aqueous suspension in rats was ca. 170% and 7461%, respectively, which was found to be significantly high in both the cases[249].

The same authors reported a further study on SLN prepared with a solid lipid and an emulsifier (2:1 monostearate:lecithin) and loaded with artemisone (10-aminoartemisinin derivative). SLN effects against human melanoma A-375 cells and human keratinocytes HaCaT were evaluated. Encapsulation efficiency was ca. 80% with average particle sizes of ca. 295 nm and a zeta potential of -12 mV. The drug release after 7 hours was 85%. The MTT assay indicates that formulation significantly suppresses melanoma cells ($P \leq 0.05$) in a dose dependent manner. At 0.06 mg/mL, free artemisone suppresses almost 50% of the melanoma cell growth, whereas at this concentration SLN almost completely suppresses melanoma cell growth[245].

Nanostructures lipid carrier

Nanostructured lipid carriers (NLC) represent a second generation of SLN, overcoming some disadvantages such as an unpredictable gelation tendency, polymorphic transition, and low incorporation due to the crystalline structure of solid lipids. NLC contain a mixture of lipid and solid phases that forms a disorganized liquid lipid matrix, which accommodates active substances increasing their stability and controlling drug extrusion.

NLC loaded with artemether (Nanoject) were formulated by employing a microemulsion template technique. The average particle size of Nanoject was ca. 63 nm and the encapsulation efficiency was found to be ca. 30%, with a sustained released. *In vitro* haemolytic studies showed that Nanoject had lower haemolytic potential (approximately 13%) as compared to all the components when studied individually. Nanoject showed significantly higher ($P<0.005$) antimalarial activity as compared to the marketed injectable oily intramuscular formulation. The antimalarial activity of Nanoject lasted for a longer duration (more than 20 days) indicating that Nanoject may be long-circulating *in vivo*. Nanoject showed significantly higher survival rate (60%) even after 31 days as compared to marketed formulation which showed 0% survival (100% mortality)[250].

The pharmacokinetic and tissue distribution after intravenous administration of DHA in solution and loaded in NLC were compared. Glycerol monostearate and Miglyol® 812 were used as solid lipid material and liquid lipid material, respectively. Surfactants were Tween 80 (1%) and Poloxamer 188 (1%). Each preparation was injected through the tail vein at a DHA dose of 10 mg/kg. Following intravenous administration of a DHA solution - the mean measured peak plasma concentration achieved was 917.51 ng/mL and for drug-loaded in NLC it was 289.28 ng/mL. After 2 h, the plasma concentration was lower for the DHA solution than that for drug-loaded in NLC because of its solubility in plasma ensuing rapid distribution, elimination and slower release of DHA from NLC leading to lower clearance. AUC ($0\sim\infty$) increased from 633.97 ng/mL/h for the drug solution to 1382.45 ng/mL/h for the drug-loaded NLC. However, the clearance decreased from 15.77 to 7.23 mg/kg/h·(ng/mL) accordingly.

The mean residence time value of the drug-loaded NLC (25.99 h) was higher than that of drug solution (0.98 h). The distribution half-life of both formulations was equal (0.06 h), while the volume of distribution of drug-loaded in NLC was 14.91 (mg/kg)/(ng/mL) and this was considerably larger than that (5.21(mg/kg)/(ng/mL)) of the drug solution. Furthermore, CLs of DHA solution was higher than that of loaded in NLC, which suggested that NLC is an effective sustained-release drug delivery system. In the tested organs, the AUC values of the formulated DHA were higher than that of the drug solution in liver, spleen, lung, brain and muscle, and lower than the drug solution in heart and kidney[251].

NLC based on glyceryl trimyristate and soybean oil and loaded with 10% artemether and surface-tailored with a combination of non-ionic, cationic or anionic surfactants were developed. Their mean particle size, zeta potential and encapsulation efficiency were ca. 120 nm, -38 mV and 97%, respectively. Haemolytic activity was within the acceptable range (7%) revealing low toxicity risk of NLC for parenteral delivery of artemether. Biocompatibility was established by hepato- and nephrotoxicity analyses. *In vivo* anti-malarial studies revealed enhanced activity of SLN formulation in comparison to a conventional plain drug solution and to a marketed formulation used to treat patients with malaria[252].

Nanoparticles

Polymeric and inorganic nanoparticles are extensively used for the encapsulation of various useful bioactive molecules and medicinal drugs. Biodegradable polymeric nanoparticles are highly preferred because they provide controlled/sustained release property and biocompatibility with tissue and cells. The drug molecules either bound to surface as

nanosphere or encapsulated inside as the most commonly and extensively used polymeric nanoparticles (poly-d,l-lactide-co-glycolide, polylactic acid, poly-ε-caprolactone, poly-alkyl-cyanoacrylates, chitosan and gelatine). Among the inorganic nanoparticles, silver, iron and gold ones are the more attractive both for diagnostic and therapeutic purposes.

A copolymer of artesunate with magnetic nanoparticles of Fe_3O_4, have been tested for their cytotoxicity on K562 cells by MTT assay, while their apoptosis rate was measured by flow cytometry. After being incubated with the copolymer of artesunate with the magnetic nanoparticles for 48 h, the growth inhibition rate of K562 cells was significantly increased compared with that of K562 cells treated with artesunate alone ($P < 0.05$). The apoptosis rate of K562 cells was increased significantly compared with that of K562 cells treated with artesunate alone, suggesting that the nanoparticles can enhance the activity of artesunate. Interestingly, caspase inhibitor Z-VAD-FMK attenuated the copolymer-induced cell death. The nanoparticle increased the expression of bcl-2, bax, bcl-rambo, and caspase-3 proteins, and decreased the expression of survivin protein in K562 cells compared with artesunate treatment alone[253].

CD derivatives grafted with decanoic alkyl chains (CD-C_{10}) yielded either nanosphere or nanoreservoir-type systems with a size ranging from 70 to 220 nm. Both types of nanostructures were able to associate artemisinin with a dosage corresponding to drug levels of 0.3 and 1.6 mg/mL, for the spherical and reservoir-type nanosystems, respectively. PEG surface-decorated nanoparticles were also prepared by co-nanoprecipitation of PEG fatty acid esters and CD-C_{10} molecules. The integration of the PEGylated amphiphiles within the CD-C_{10} nanostructures did not influence the artemisinin bioavailability. Both types of artemisinin-loaded nanosystems showed a sustained *in vitro* release profile over 96 h (nanoreservoirs) and 240 h (nanospheres). Finally, the *in vitro* antimalarial activity was evaluated using the lactate dehydrogenase assay. The formulations inhibited the growth of cultured *Plasmodium falciparum*, both multi-resistant K1 and susceptible 3D7 strains with IC_{50} values (2.8 and 7.0 ng/mL close to those of reference artemisinin solution[254].

A paper reported on the preparation and characterization of chitosan/lecithin nanoparticles (below 300 nm) loaded with artesunate and artesunate complexed with β-cyclodextrin to boost the antimalarial activity. Drug entrapment efficiency was found to be maximum (90%) for nanoparticles containing 100 mg of artesunate. Increased *in vivo* antimalarial activity in terms of parasitemia values was observed in infected *Plasmodium berghei* mice after the oral administration of all the prepared nanoparticle formulations[255].

DHA was encapsulated in gelatine or hyaluronan nanoparticles (30-40 nm). The entrapment efficiencies for dihydroartemisinin were approximately 13 and 35% for the gelatine and hyaluronan nanoparticles, respectively. The proliferation of A549 cells was inhibited by both nanoparticles. Fluorescent annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) staining displayed low background staining with annexin V-FITC or PI on DHA-untreated cells. In contrast, annexin V-FITC and PI stains dramatically increased when the cells were incubated with gelatine and hyaluronan nanoparticles, with an increased anticancer proliferation activity than DHA alone in A549 cells[256].

Fe_3O_4@C/Ag@mSiO_2 nanoparticles loaded with 484 mg artemisinin/g, demonstrated pH-responsive Fe^{2+} release and the concentration of Fe^{2+} ions reached 2.765 nmol/L in HeLa cells. The antitumor efficacy of ART-loaded FCA@mSiO_2 nanoparticles measured by MTT assay was significantly enhanced compared with free artemisinin. It was suggested that the ART-loaded FCA@mSiO_2 nanoparticles are internalized by HeLa cells and located at the acidic compartments of endosomes and lysosomes, releasing Fe^{2+} ions to non-enzymatically convert artemisinin to toxic products for killing cancer cells[257].

Artemisinin-loaded poly lactic co-glycolic acid (ALPLGA) nanoparticles nanoparticles were ca. 221 nm in diameter, with polydispersity index, zeta potential, drug loading and entrapment efficiency of ca. 0.1, -9.07 mV, 28 % and 68 %, respectively. Atomic force microscopy and transmission electron microscopy studies indicated that the particles were spherical in shape. The drug release behaviour investigated by dialysis method at pH 7.4 and 5.5 exhibited a biphasic pattern characterized by the initial burst release during the first 24 h, followed by a sustained release up to 100 h. Nanoparticles were stable for a period of about one month at 4°C, and no significant changes ($P > 0.05$) were observed in the mean particle size, PDI, zeta potential and drug loading of the nanoparticles. Investigation of toxicity of the nanoparticles on murine macrophages revealed no significant toxicity, while native artemisinin exhibited significant toxicity at 200 μg/ml with a drop in viability of cells to 40%. The pentamidine that served as a standard anti-leishmanial drug also indicated signs of toxicity on the murine macrophages. The nanoparticles significantly inhibited the growth of intracellular amastigotes compared to free artemisinin where as empty nanoparticles did not exhibit any anti-leishmanial activity. The IC_{50} value of nanoparticles for intracellular amastigotes, calculated by linear regression analysis was found to be 2.9-fold lower than free artemisinin (11.9 *versus* 3.93 μg/ml). Treatment of amastigote-infested macrophages with ALPLGA nanoparticles also showed a significant reduction in the percentage of infected macrophages resulting in 3.6-fold lower IC_{50} value compared to free artemisinin (14.86 *versus* 4.16 μg/ml)[258].

Albumin-based nanoparticles were developed as carriers of artemisinin. Their mean diameter was 339 nm, while the zeta potential was -43.8 mV. When artemisinin was loaded to the nanoparticles with a ratio 1:10 with respect to albumin, rods were formed as revealed by transmission electron microscopy. These freeze dried nanoparticles had a mean diameter of 612 nm with an efficiency of 97.5% of entrapped artemisinin in the nanoparticles. Reconstituted nanoparticles showed satisfactory physical stability when stored at 4°C for four days. The increase in the mean diameter was 5.8% with good homogeneity (PI < 0.25). The high zeta potential values (-43.8 mV) may account for this stability by providing

sufficient electrostatic repulsion thus preventing particle aggregation. Artemisinin showed good chemical stability within nanoparticles both in its powdered (lyophilized) and reconstituted aqueous forms. After storage of lyophilized NPs for 1 month at 4°C, the percentage of artemisinin remaining in NPs was 98.4%. The antiplasmodial activity of the developed formulation was tested on *in vitro* model of chloroquine-resistant strain of *P. falciparum* (FcB1). This revealed improved activity compared with unformulated artemisinin (IC_{50} was <3.5 *versus* 11.4 nM). The *i.v.* route in humanized mice infected with the human parasite *P. falciparum* (3D7 strain) assessed the *in vivo* antimalarial activity of the developed formulation. A 4-day treatment with 10 mg/kg of nanoparticles achieved a sharp reduction in parasitemia (96%) measured the day after the end of the treatment. Moreover, mice survived for more than 18 days with no recrudescence till the end of the experiment[259].

Poly(lactic-co-glycolic acid) (PLGA) nanoparticle coated with a 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) were co-loaded with DHA and doxorubicin for combination chemotherapy. Doxorubicin was conjugated with PLGA with an efficiency of 56% and nanoparticles were prepared adding 0.5% 4-(dimethylamino)benzaldehyde (DMAB) and characterised (diameter was ca. 150 nm and zeta potential was ca. +50 mV, polydispersity index 0.13), loading content of doxorubicin was ca. 0.54%. In a second step, DHA was solubilized in an organic solvent with DPPC and used to coat the nanoparticles. As a result nanoparticles had a core-shell structure self-assembled via electrostatic forces between the cationic DMAB-modified polymer particle and the zwitterionic DPPC lipid. The physicochemical properties of the dual drug-loaded core-shell-type lipid/particle assemblies were characterized (diameter was ca. 220 nm and zeta potential was ca. -12 mV, polydispersity index 0.15) loading content of doxorubicin was ca. 0.23%, while that of DHA was ca. 1.95%. *In vitro* release profile at pH 7.4 revealed that nanoparticles can generate a systemic cytotoxic effect with DHA followed by a delayed doxorubicin cytotoxic effect. The results of colorimetric cell viability assay evidenced a synergistic effect of drugs and cellular uptake experiments demonstrated that the lipid/particle hybrid could increase doxorubicin accumulation in cell nuclei, thus enhancing cell cytotoxicity[260].

Artemisinin-loaded poly lactic co-glycolic acid (PLGA) nanoparticles prepared with a particle size of ca. 220 nm, 29% drug loading and 69% encapsulation efficiency. When administered at doses of 10 and 20 mg/kg body weight showed superior anti-leishmanial efficacy compared with free artemisinin in BALB/c model of visceral *Leishmania*. There was a significant reduction in hepatosplenomegaly as well as in parasite load in the liver (ca. 85.0) and spleen (ca. 82.0) with nanoparticles treatment at 20 mg/kg body weight compared to free artemisinin (ca. 70% in liver and 63% in spleen). In addition, nanoparticle treatment restored the defective host immune response in mice with established visceral *Leishmania* infection. The protection was associated with a Th1-biased immune response as evident from a positive delayed-type hypersensitivity reaction, escalated IgG2a levels, augmented lymphoproliferation and enhancement in pro-inflammatory cytokines (IFN-γ and IL-2) with significant suppression of Th2 cytokines (IL-10 and IL-4) after *in vitro* recall, compared to infected control and free artemisinin treatment[261].

Nanotubes and fullerenes

Nanotube is a tube-shaped material, made of carbon, having a diameter measuring on the nanometer scale ranging from <1 nm up to 50 nm. The graphite layer appears somewhat like a rolled-up chicken wire with a continuous unbroken hexagonal mesh and carbon molecules at the apexes of the hexagons. They include multiwall nanotubes, single wall and double wall nanotubes. In addition, classical fullerenes are made of carbon, cage-like, hollow molecules of pseudo-spherical symmetry consisting of pentagons and hexagons only, resulting in a trivalent (and in the most ideal case) convex polyhedron with exactly three edges (bonds) joining every vertex occupied by carbon. The unique physicochemical properties of both nanotubes and fullerenes with easy surface modification they have promising applications both in diagnostic and nanomedicine applications.

A multi-functional tumor-targeting drug delivery system employing hyaluronic acid-derivatized multi-walled carbon nanotubes, transferrin as targeting ligand and artemisinin as a model drug for cancer treatment was constructed. This delivery system not only retained cytotoxicity of artemisinin, but also demonstrated synergistic anti-tumor effect using artemisinin and transferrin. Compared with free artemisinin, remarkably enhanced anti-tumour efficacy of this drug vehicle was found both in cultured MCF-7 cells *in vitro* and in a tumor-bearing murine model *in vivo*, due to increased intracellular accumulation of artemisinin and co-delivery of transferrin and artemisinin analogues. The formulation with laser irradiation demonstrated the highest inhibition effect compared to the other groups[262].

In a further study, hyaluronic acid was grafted onto fullerene to get a water-soluble biomaterial and then combined with transferrin to obtain a multi-functional drug delivery system with significant tumor-targeting efficacy and powerful photodynamic therapy capacity. Artesunate was adsorbed on the developed multifunctional vector with a high loading efficacy. Compared with free artesunate, remarkably enhanced antitumor efficacy of fullerenes was realized both in a cultured MCF-7 cells *in vitro* and in a tumor-bearing murine model *in vivo*, due to increased intracellular accumulation of artesunate in tumor and activated mechanism by co-delivery of transferrin and artesunate. Furthermore, after laser irradiation, the relative tumor volume of functionalized fullerenes declined by half, from ca. 1.72 to ca. 0.84[263].

Conclusions and perspectives

Artemisinin-containing herbs have been used for centuries in TCM. Artemisinin was rediscovered for the treatment of malaria by Youyou Tu. Recently, scientists have synthesized a large array of monomeric and dimeric analogues using artemisinin structure as a scaffold. Artemisinins display

anti-infective effects against a broad spectrum of pathogens, including causative agents associated with infections in immunocompromised hosts such as herpes viruses, cytomegaloviruses, beside hepatitis viruses B and C, and the pathogenic yeast *Cryptococcus neoformans*.

Motivated by the existence of the metabolic instability of the acetal-derived artemisinins, scientists have designed and synthesized the second generation of artemisinins, the C-10 carba non-acetal counterparts. The non-acetal analogues proved metabolically stable and orally bioavailable. Together with their anti-*Plasmodium* properties, scientists around the world have investigated their activities against cancer, viruses, bacteria, fungi, trematodes and other protozoans with successful results. At present, artemisinins emerged with multi-functionality against a wide range of human, animal and plant pathogens without severe side effects on humans, even in immuno-compromised populations such as pregnant women and children.

Added to this, exploitation of the advancement in pharmaceutical technology, using nanotechnology and innovative formulations, has led to significant improvement of the stability, bioavailability, pharmacokinetic profile and reduction of the toxicity of the acetal artemisinins such as artemisinin, arteether, artemether and artesunate as well as derivatives of artemisinin-piperazine dimers, and artemisone.

In spite of all advances in the treatment of artemisinin-sensitive pathogens, the emergence of resistant strains remains a serious problem. There is still the need and the possibility to further expand the basic and translational investigation to enrich the armamentarium to combat diseases caused by different types of pathogens, in addition to malaria, based on artemisinin and its derivatives and/or novel formulations, alone or in combination with other pathogen-specific drugs.

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