

Is Artesunate the Best Partner of Gemcitabine in Pancreatic Cancer?

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ABSTRACT

Pancreatic adenocarcinoma (PDAC) is still one of the most malignant and difficult to treat cancers. The therapeutic protocols in use, such as gemcitabine, gemcitabine associated with nab-paclitaxel and/or cisplatin or the FOLFIRINOX scheme have added very little to PDAC outcome. It is clear by now, that none of them can do the job alone. The more than 3,300 trials registered in clinicaltrials.gov is the best proof that research has not yet found an adequate response to tackle this disease. Thus, an innovative search is badly needed. As part of this investigation we came across a phytotherapeutic product that has been very successful for the treatment of falciparum- and vivax- caused malaria: artemisinin derivatives. These derivatives showed very low toxicity for humans and have been tested in millions of patients with paludism.

Interestingly, they have also shown important anti-cancer properties. Regarding PDAC in particular there is strong evidence supporting not only an additive effect to gemcitabine without a concomitant increase in human toxicity, but also decreased resistance. This mini-review will discuss the evidence showing that artemisinin derivatives can be the best possible association with gemcitabine for PDAC chemotherapeutic treatment.

Keywords: Artemisinin, artesunate, dihydroartemisin, pancreatic cancer, ferroptosis, PDAC.

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I. INTRODUCTION

Exocrine pancreatic cancer and pancreatic ductal adenocarcinoma (PDAC) in particular, is one of the most difficult malignancies to treat, with poor therapeutic response and short overall survival [1]. The incidence of pancreatic cancer is on the rise [2], [3]. The tumor becomes resistant to gemcitabine and to the FOLFIRINOX scheme, usually the first line protocols [4], [5], very soon. Thus, a new approach is urgently needed. Consulting the clinicaltrials.gov internet page (April 2022) using pancreatic cancer as the search criteria, 3,172 studies in different phases were found. If the search is restricted to pancreatic adenocarcinoma we find 1073 trials. Such an abundance of research may be clear proof that we are still far away from an effective approach. Treatment of different cancers has significantly improved in the last twenty years. This is not the case of PDAC. The anti-metabolite gemcitabine is the gold standard for the first line treatment; however, the response rate is low and overall survival has only improved minimally. The main reason for this failure is intrinsic or early-acquired resistance. It is quite evident that gemcitabine alone is unable to do the job. 911 clinical trials (clinicaltrials.gov) and the very low 5 year survival are the proof of concept that gemcitabine needs to be improved somehow. On the other hand, there is a group of drugs that have been highly successful in the treatment of

malaria, artemisinin derivatives, which have shown significant anti-cancer activity *in vitro* and *in vivo*. Interestingly, these drugs were also found to be active against pancreatic cancer. The aim of this review is to analyze and discuss a possible partnership between artemisinin derivatives and gemcitabine for the treatment of pancreatic cancer.

II. ARTEMISININ DERIVATIVES

A. Historical Background

“Take one bunch of Qinghao, soak in two sheng of water, wring it out to obtain the juice and ingest it all”, this comes from *The Handbook of Prescriptions for Emergency Treatments* for intermittent fever therapy. The interesting point is that this handbook was written in 340 AD by Ge Hong (also known as Ko Hung 283-343 AD), and intermittent fever was the ancient term used for malaria. But even more interesting is that such an old remedy and its description was fished out of Traditional Chinese Herbal Medicine and was studied in depth by a Chinese phytochemist Tu YouYou in the 1970s as part of a top-secret Chinese government project (Project 523) for the treatment of malaria [6], [7]. For a detailed description of how the discovery took place, read [8].

The work of Tu YouYou's team finally led to the discovery of artemisinin (ART) and its derivatives (ARTs), which are

considered the most powerful anti-malarial drugs available today. In spite of this major discovery, according to modern Western standards, traditional Chinese herbal therapy is considered to use inadequate methodologies, and its effectiveness poorly documented [9]. Undoubtedly, ART represents the exception. Reference [10] described how this rediscovery of artemisinin happened: her team investigated more than 2,000 Chinese herbal preparations identifying 640 possible candidates for anti-malarial activity. *Artemisia annua* extracts showed promising results but they faced problems in the purification and extraction methods, until they found the above-mentioned *Handbook of Prescriptions for Emergencies*. The form of preparation described by Ge Hong gave Tu YuoYuo the idea that the problems she found were due to the excessive heat employed in the extraction process which destroyed the active components. This time they used a “cold” extraction method, and ART was reborn against malaria [10]. Traditional Chinese medicine has successfully used the herb *Artemisia annua* against malaria since ancient times [11].

Nowadays, ARTs are well established anti-malarial agents with excellent safety profiles. Artemisinin-based combination therapies are recommended by the World Health Organization (WHO) as the first-line treatment for uncomplicated falciparum malaria in all areas where the disease is endemic [12] (updated in 2022 [13])[14]. ART and ARTs have shown interesting anti-cancer effects that are in the initial stage of clinical evaluation.

B. Chemistry

ART was isolated and purified in 1972 and the chemical structure became known in 1979. The amount of ART obtained from *Artemisia annua* is very low (less than 0.1%). ART is poorly soluble in oil and water, and it has a short plasma life. Therefore, nowadays ART is not the drug used to treat malaria, because it has been replaced by its derivatives which are more hydrophilic or more lipophilic. They are obtained from the parent compound and are 8 to 10 times more effective in the treatment of malaria [15]. ART derivatives like artesunate (ARS) and dihydroartesunate (DHA) are hydrophobic and partition into biological membranes; arthemeter and artheeter are oil soluble while sodium artesunate and sodium arteminate are water soluble. (For an extensive review of the chemistry of ARTs read [16]. Ultrastructural studies of parasites treated with ART and its derivatives show that the drug is mainly present in the parasite membranes together with alterations in ribosomal organization and endoplasmic reticulum. The affected membranes include the limiting membrane, digestive vacuole membranes, the nuclear envelope, endoplasmic reticulum, and mitochondrial membranes. Thus, the ARTs localize in specific parasite membranes and lead to a total disorganization of the parasite's ultrastructure including disappearance of ribosomes with protein synthesis inhibition [17], [18].

Artemisinin is a sesquiterpene lactone with an endoperoxide. The endoperoxide bridge is essential for ART's cytotoxic activity [19]. The carbonyl group of artemisinin can be reduced to form a more soluble compound: dihydroartemisin (DHA). DHA is the scaffold on which many derivatives have been synthesized.

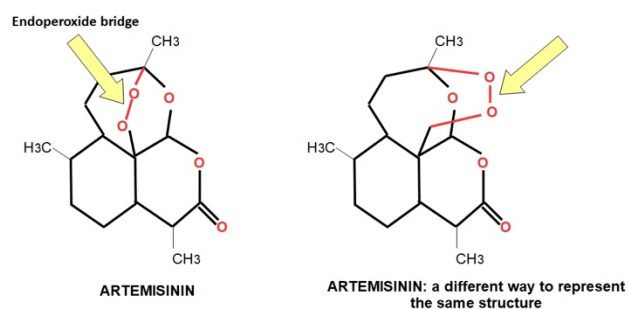


Fig.1. Chemical formula of artemisinin showing the endoperoxide bridge.

C. Mechanism of Action

To understand how ART and ARTs might work in cancer, it is necessary to examine first how they work against *Plasmodium falciparum* and *vivax*, and related parasites, although this mechanism of action has not been fully elucidated. The endoperoxide link and heme iron are key players in ART and ARTs' anti-malarial actions. The clearest and simplest mechanism of action was proposed by [11]. They suggested that the activation of ART was triggered by iron molecules generating toxic free radicals.

Reference [20] determined that endoperoxides like ART and its derivatives interfere with the plasmodial hemoglobin catabolic pathway and inhibit heme polymerization. The endoperoxide ring was essential to this activity.

The mechanism of free radical formation seems to be a two-step process:

- The iron contained in heme molecules breaks down the endoperoxide structure of ART generating an oxy free radical which then produces a carbon-centered free radical;
- This carbon-centered free radical acts as a protein alkylating agent interfering with essential proteins [21], [22].

ART and its derivatives also inhibit exported protein 1 (EXP1), a membrane glutathione S-transferase [23]. There are controversial reports about ART and derivatives targeting SERCA 1a. (sarcoplasmic/endoplasmic reticulum Ca²⁺-ATPase 1a) [24], [25].

In summary the proposed mechanisms for the anti-malarial actions of ARTs, are [26], [27]:

- The heme-iron hypothesis explained above;
- Reaction with a histidine-rich protein of parasites;
- SERCA-1a inhibition;
- Disruption of mitochondrial membrane potential.

In [28] discussed the mechanism of action of artemisinin derivatives along the above lines, but no clear conclusion was reached and they stated that: “The debate continues”. The debate continued until 2012 when [29], [30] explained a new cell death mechanism: ferroptosis.

1) Ferroptosis

Ferroptosis is a regulated form of cell death dependent on iron and characterized by lipid peroxidation that produces an accumulation of lipid peroxides leading to cell toxicity and death which is different from apoptosis. This is a consequence of the decreased/lost activity of the enzyme glutathione peroxidase 4 (GPX4) that is unable to repair/prevent lipid peroxidation leading to the formation of toxic products such as lipid-based reactive oxygen species

[31], [32].

Artemisin and its derivatives have been found to be ferroptotic inducers [33].

Although ferroptosis is one of the main mechanisms involved in cancer cell killing by ARS, it is not the only one.

2) Mechanisms Involved in Anticancer Effects

The mechanisms that may explain the inhibitory action on cancer progression by ARTs have been postulated:

- Ferroptosis through a similar mechanism found against Plasmodium genus organisms, ART and derivatives, with the intervention of intracellular iron molecules, produce carbon-centered free radicals with cytotoxic effects [34]. This would explain apoptosis in the malignant cells with minor adverse events in normal cells, because while the intracellular content of iron is high in tumors [35], [36], it is significantly lower in normal cells. This also means that malignant cells that overexpress transferrin receptor molecules are particularly susceptible to ART and derivatives [37]. Furthermore, high expression of hepcidin and/ or low expression of ferroportin, both situations lowering intracellular iron, are signs of poor prognosis in pancreatic cancer [38]. However, this mechanism seems insufficient to explain all the anti-cancer effects of ART. In summary Artemisinin derivatives kill cells by reacting with iron to form free radicals, and iron plays a double role by favoring tumor progression [39] but on the other hand killing the cell when artemisinin derivatives are administered.
- Anti-angiogenesis [40]-[45]. The mechanism seems to be down-regulation of VEGF (vascular endothelial growth factor) and vascular endothelial growth factor receptor [46].
- Anti-lymphangiogenesis by suppression of VEGF-C [47].
- Interference or inhibition of pro-proliferative proteins such as:
 - a) Interference with Sp1 binding to the CDK 4 gene promoter decreasing CDK4 transcription [48].
 - b) Down-regulation of the transcription factor E2F1 which interferes with CDK2 promoter activity [49].
 - c) Alkylation of other significant proteins (approximately 5 to 18% of ART added to a cell culture bound with catalase, cytochrome c and hemoglobin but not to DNA) [50].
- Down-regulation of matrix metalloproteinase-9 (MMP-9). MMP-9 is not only produced by cancer cells, but also by macrophages and fibroblasts adjacent to the tumor. The extracellular matrix metalloproteinase inducer (EMMPRIN), also known as CD147 or basigin, located on the surface of tumor cells induces adjacent macrophages, fibroblasts, and endothelial cells to produce MMPs. ART and ART derivatives down-regulate EMMPRIN and therefore decrease MMP-9 production [51].
- Down-regulation of the Wnt/ β catenin pathway. The Wnt/ β catenin pathway plays an important role in colorectal cancer due to the loss of the APC gene [52], but its role in PDAC has not been clearly

established [53]. However, it is one of the players in pancreatic cancer resistance [54], [55]. Artesunate has shown abilities to down-regulate the Wnt/B catenin pathway in gastrointestinal tumors [56].

- Immunomodulation. ARTs decrease immunosuppression in CRC cells by decreasing TGF- β 1 and IL-10 [57].
- Inhibition of mTORC1 signaling in rhabdomyosarcoma at clinically achievable concentrations [58] and also in nasopharyngeal carcinoma [59].
- DNA genotoxic activity. ARTs produce double strand breaks [60], [61] due to oxidative stress.
- Decreasing the mitochondrial membrane potential and intracellular ATP concentration in various cell lines in a dose dependent manner [62] thus modulating multidrug resistance at the mitochondrial/apoptotic level, but not at the P-gp or MRP1 in adriamycin-resistant cells.
- ARTs anti-tumoral activity is not modified by the expression of MDR proteins. ARTs treatment of multidrug-resistant cells expressing MDR1, MRP1, or BCRP showed that there was no cross-resistance with other chemotherapy drugs [63].
- Inhibition of the PI3K/AKT axis. Artesunate was found to inhibit this axis in experimental allergic asthma [64], human rheumatoid arthritis [65], human cervical carcinoma [66], oral squamous cell carcinoma [67].
- Inhibition of complex I and II of the electron transport complex, thus generating important oxidative stress [67] which is proportional to the oxidative metabolism present in the tumor.
- Inhibition of lipid synthesis. [68] showed that ARTs were able to inhibit fatty acid synthesis in colorectal cancer cells.

Due to all these effects, and the minimal toxicity for normal cells ARTs can be considered cancer chemotherapeutic drugs [69].

III. ARTIMISININ DERIVATIVES IN CANCER

In 1993, [70] working with Ehrlich ascitis cells were the first to describe the cytotoxicity of artemisinins on tumor cells. However, there are earlier Chinese publications in this same sense [71]. In 1994, [72] tested the cytotoxic activity of different compounds isolated from *Artemisia annua* *in vitro*. He found that ART showed a significant activity against 5 different tumor cell lines. This was the beginning of the idea that ART may have anti-cancer activity [73].

Anti-cancer effects were confirmed in different tumors such as:

A. Oral Squamous Cancer Cells

ART and its derivatives were tested against oral cancer cells by [74] who found that deoxoartemisinin trimer had the most potent cytotoxic and growth inhibiting effect, which was even more powerful than paclitaxel, 5FU and cisplatin on YD-10B oral cancer cells.

B. Laryngeal Squamous Cell Carcinoma

There is a case report of a patient with squamous cell laryngeal carcinoma with lymph node metastasis who was treated with ARS (IV and oral) and ferrous sulfate. The tumor and the lymph nodes significantly decreased in size after two months of treatment and the patient was stable after 9 months [75].

C. Leukemia Cells

Myeloid leukemia K562 cells underwent autophagy and cell cycle arrest when treated with DHA. Iron was an essential component for these effects [76]. Artenuin B derivatives are active against leukemia P 388 cells *in vitro* [77]. In acute myeloid leukemia cells, ART and DHA showed selectivity against mixed lineage leukemia rearrangements and mutations of FLT3-ID. Synergy with ARA-C was also observed [78], and ART derivative dimers were particularly powerful against leukemia cells [79]. Furthermore, ART was able to induce ROS-mediated apoptosis in doxorubicin-resistant leukemia cells [80] and reduce stemness [81].

D. B Cell Lymphoma (CD20 +) Cells

ARTs showed synergistic cytotoxic effects with rituximab [82], and suppressed growth of malignant cells without affecting the normal ones, by inducing a powerful endoplasmic reticulum stress [83]. Lymphoma and myeloma cells are highly sensitive to ARTS-induced apoptosis [84].

E. Prostate Cancer Cells

DHA and two different dimers were used on prostate cancer cell lines. The C4-2 and LNCaP cell lines showed increased apoptosis and growth arrest. No significant changes were observed in DU 145 cells [85]. Reference [48] showed that the mechanism of action in prostate cancer cells was due to interference with the Sp1 transcription factor and [86] found that artesunate was able to inhibit the androgen receptor.

F. Breast Cancer Cells

MCF7, an estrogen responsive cell line, treated with ART showed decreased progression of the hormonally induced estrogen-stimulated cell cycle. ART also down-regulated ER α protein and transcripts and it acted synergistically with fulvestran [87]. ART inhibited proliferation and produced G1 cell cycle arrest in these cells. Proliferative proteins of the cell cycle (CDK2, CDK4, cyclin E, cyclin D1 and E2F1 transcription factor) were all decreased [88]. ART was also effective against triple-negative breast cancer cell lines [89]. Reference [90] showed that ARS cytotoxic activity on breast cancer cells was a consequence of apoptosis induced by the inhibition of HSP70 ATPase.

G. Lung Cancer (NSCLC)

DHA has shown marked activity against the human lung adenocarcinoma cell line A549, *in vitro* and *in vivo* [91]. Akt/GSk3 β /cyclin D1 was one of the down-regulated pathways [92]. ARS increased radiosensitivity of NSCLC [93] and decreased EGFR expression [94]. Reference [95] described ARS effects against migration, invasion, and metastasis *in vivo* and *in vitro*: decreased expression of MMP-2 and MMP-7 and uPA promoter/enhancer actions. *In vivo* metastases were considerably diminished. Inhibition of

autophagy with chloroquine increased apoptosis of lung cancer cells *in vitro* by ROS increase [96]. A clinical experiment with 120 patients (60 treated with ARS plus vinorelbine and cisplatin and 60 with the same treatment but without ARS) showed no significant difference between the two groups in short-, medium-, and long-term survival, but there was a significant difference in progression-free survival which was higher in the ARS treated group [97].

H. Melanoma

Two cases of metastatic uveal melanoma were treated with ARS as adjuvant treatment. Neither of the two cases responded to standard chemotherapy. One of the patients presented a temporary response, but the other showed a regression of metastasis and was alive after 47 months [98]. ART decreased human melanoma cell migration through down-regulation of alpha V beta 3 integrin and metalloproteinase-2 [99]. DHA targets metastatic melanoma cells by up-regulating the apoptotic protein NOXA without affecting normal melanocytes [100]. ARS decreased melanoma progression by blocking STAT3 signaling [101] and inhibited vasculogenic mimicry in choroidal melanoma [102].

I. Metastatic Renal Cell Carcinoma (mRCC)

When ARS was tested against different human RCC cell lines it showed potent cytotoxicity and increased the malignant cells' sensitivity to sorafenib *in vitro*. Cytotoxicity correlated with expression of transferrin receptors [103]. Furthermore, ARS inhibited growth of sunitinib-resistant renal cancer cells [104], [105]. Importantly, artesunate showed antiangiogenic effects which is an important factor in a tumor such as mRCC which is highly dependent on angiogenesis [106].

J. Hepatocarcinoma

Reference [107] tested ART and derivatives against different lines of hepatoma cells (HepG2, Huh-7, BEL-7404, and Hep3B), and a normal human liver cell line, and found G1 arrest in malignant cells. They observed greater cytotoxicity in hepatoma cells and markedly less in normal cells. Proliferation was significantly decreased with G1 arrest, decreasing all proteins related to proliferation and a significant increase in pro-apoptotic proteins. ART and DHA inhibited tumor growth in mice implanted with HepG2 and Hep3B xenograft tumors. These results were achieved in normal and altered P53 cells. The important issue is that ART and derivatives were toxic for malignant cells but did not affect the normal hepatic cells used as controls. Cytotoxicity for hepatocarcinoma cells has been confirmed by many authors [108]-[119].

K. Colorectal Cancer (CRC)

There is strong evidence at different levels (laboratory and clinical settings) showing ARTs beneficial effects in colorectal carcinoma. In a double-blind randomized trial with artesunate given preoperatively to 20 patients with CRC (9 received artesunate and 11 in the control group were given placebo), after a median follow up of 42 months only 1 patient in the artesunate group showed recurrence, while 6 patients in the placebo group had a relapse [120]. The number of patients included in this protocol was small, and the drug was

only administered during the two weeks before surgery. Nevertheless the difference in the recurrence rate between treated and placebo controls is quite significant. At the clinical level, this experiment, showed not only a decrease in recurrence rate in patients receiving preoperative ARS but also that all the patients who were given ARS in the preoperative period were alive at the time the paper was published while mortality in the control group was 30%. At the cell level, [121] found that ARS decreased the growth of human colorectal carcinoma and inhibited the over-expressed Wnt/ β -catenin pathway. DHA reduced colorectal cancer cell viability in a significant manner inducing accumulation of ROS (reactive oxygen species) and apoptosis through the mitochondrial-caspase pathway [122]. Another mechanism involved in DHA-dependent apoptosis is endoplasmic reticulum stress which results from SERCA inhibition [123]. Reference [63] found that the highest responsiveness to ARTs anti-cancer activity was in colon cancer and leukemia cell lines. Colon cancer cells have the ability to produce immunosuppression which disables natural immunologic defenses. Artesunate can reverse this process in colon cancer cell lines (Colon26 and RKO colorectal cancer cells) by decreasing expression of TGF- β 1 and IL-10 [57]. There is a relation between drug sensitivity and colon cancer phenotype: the more undifferentiated tumors are more susceptible to ARS cytotoxicity. It is a well accepted concept that hypoxia is a common cause of treatment failure and resistance to chemoradiotherapy. DHA applied to colorectal cancer cells showed that it was able to induce apoptosis even under hypoxic conditions. Mitochondrial cytochrome c pathway was the apoptotic direction induced by DHA under normoxic conditions whereas under hypoxia, DHA induced a caspase-independent apoptosis-like cell death. Recently, [124] synthesized new thymoquinone-artemisinin hybrid molecules, one of which had strong activity, even superior to the effects of 5-FU, against colorectal cancer cells.

L. Retinoblastoma

Reference [125] found ART specific cytotoxicity against RB cells, with low toxicity in normal retina cells.

M. Ovarian Cancer

Reference [126] synthesized a melfalan-artemisinin conjugate that showed significant cytotoxicity against ovarian cancer cells and had low toxicity against normal cells. The cytotoxicity of the conjugate was significantly superior to that found with each component separately. Reference [127] found that DHA inhibited the proliferation of ovarian cancer cells *in vitro*, and also reduced migration and invasion. There was also a significant reduction of metastasis *in vivo* in a xenograft mouse model.

N. Glioblastoma

DHA showed synergistic action with temozolomide on 10 different glioblastoma cell lines. The mechanism of action in these cases seemed induction of autophagy. This activity was observed *in vitro* and *in vivo* [128]. ARS increased the cytotoxic effect of temozolomide in glioblastoma cell lines [129], and sensitized cells to radiotherapy [130]. DHA inhibited glioma invasiveness [131].

O. Thyroid Carcinoma

Reference [132] found strong anti-proliferative effects of ARS in medullary thyroid carcinoma cells and in chemo resistant anaplastic thyroid cancer [133]. It inhibited proliferation, migration, and invasion by targeting the P13K/AKT pathway [134].

P. Gastric Cancer

ART showed inhibition of proliferation of gastric cancer cells with up-regulation of p53, p27 kip1 and p21 kip1 [135]. It also showed growth inhibition and apoptosis [136] by inhibiting COX2 [137].

Q. Osteosarcoma Cells

ARTs reduce proliferation and metastasis while increasing apoptosis [138], [139]. ARS showed selective activity against osteosarcoma *in vivo* and *in vitro* through the intrinsic apoptotic pathway and in a dose- and time-dependent manner [140]. DHA was active against human [141] and canine [142] osteosarcoma cell lines. Inhibition of the Wnt/ β catenin pathway plays a role in DHA anti-cancer activity in osteosarcoma cells [143].

R. Rhabdomyosarcoma

Reference [144] found that DHA inhibited proliferation and showed a pro-apoptotic effect in rhabdomyoma cells blocking the mTORC1 signaling pathway at clinically achievable concentrations. Similar effects were found in embryonal rhabdomyosarcoma cells *in vivo* [145].

S. Kaposi's Sarcoma

ARS inhibited growth and angiogenesis *in vivo* [146]. Kaposi's sarcoma is of endothelial origin and artesunate inhibited growth of Kaposi's sarcoma cell cultures and of the HUVECs (human vascular endothelial cells) used as controls. But while Kaposi's sarcoma cells underwent apoptosis, HUVECs did not. Artesunate also inhibited the growth of Kaposi's cells xerografted into nude mice.

T. Gall Bladder Cancer Cells

ARS decreased proliferation and increased apoptosis (increasing mitochondrial C cytochrome release) in gall bladder cancer cells. p-ERK1/2, CDK4 and cyclin D1 were down-regulated [147].

The evidence discussed above shows that ARTs exert anti-cancer effects in all kinds of malignant tissues. This long, however, incomplete list of anti-cancer activities exerted by ARTs deserves research on its potential effects on pancreatic cancer, where therapeutic resources are feeble and where new treatment options are so needed.

Only ten clinical trials with ART derivatives in cancer were found at www.clinicaltrials.gov as of April 2022.

It is of note that no studies were found regarding pancreatic cancer.

IV. ARTEMISIN DERIVATIVES AND PANCREATIC CANCER

In this regard, ARTs have shown substantial experimental activity against pancreatic cancers and may not only be an interesting complement to treatment, but a player in reversing resistance as well.

There is abundant evidence of the potential benefits of

ARTS in pancreatic cancer treatment:

1. In 2009, [148], [149] showed that DHA had the ability to inhibit growth of pancreatic cancer cells *in vitro* and *in vivo* (subcutaneous BxPC3 cells in mice). DHA reduced the ratio of Bcl-2/Bax and increased caspase-9 activation thus inducing apoptosis in a dose-dependent manner.
2. In a further study [150], this same group confirmed their findings and showed that DHA inhibited NF- κ B nuclear translocation and DNA binding in a dose-dependent manner.
3. These findings were further confirmed by [151], namely NF- κ B pathway down-regulation and apoptotic effects of DHA in pancreatic cancer *in vitro* and *in vivo*. Importantly, they found that DHA potentiated gemcitabine's anti-tumor effects. According to the authors, this may be produced by the fact that DHA prevents gemcitabine-induced NF- κ B activation.
4. Anti-angiogenesis was also one of the mechanisms described in its action on the pancreas [152].
5. Cooperation between gemcitabine and DHA achieving additive effects was found by [153] *in vitro* and *in vivo*.
6. DHA induced apoptosis through up-regulation of the death receptor 5 (DR5) [154]. Compared with single-agent treatment, DHA associated with Apo2L/TRAIL increased therapeutic efficacy enhancing apoptosis *in vitro*. Significant reactive oxygen species (ROS) generation was involved in the process. Probably lipoxidation and ferroptosis were also involved, however, at the time of this report this concept was not fully known.
7. Confirming ART's ability to induce growth arrest and apoptosis in pancreatic cancer cells Reference [155] added interesting new findings. It
 - (a) was more effective in poorly differentiated cells;
 - (b) enhanced gemcitabine's cytotoxicity;
 - (c) significantly inhibited topoisomerase II α ;
 - (d) activated caspase 3 and 7;
 - (e) down-regulated ribonucleotide reductase M2, a frequent player in gemcitabine resistance;
 - (f) up-regulated GADD53 (DNA-damage-inducible transcript 3) which plays an important role in growth arrest and apoptosis;
 - (g) down-regulated PCNA (proliferating cell nuclear antigen);
 - (h) up-regulated the pro-apoptotic gene NAG1 (NSAID activated gene 1).ARTs concentrations used in the experiments mentioned above are all within the range that can be achieved in patients treated for malaria [156].
8. Reference [157] found that artesunate was able to induce ferroptosis in pancreatic cancer cells. The drug did not affect normal pancreatic cells and cytotoxicity was higher in constitutively-active KRAS cell lines. Furthermore, ferroptosis inhibitors abrogated cell death. Importantly, KRAS is usually a driver gene/protein of PDAC (95% of mutated active KRAS is found in PDACs) [158]. Sotorasib has been FDA approved as a KRAS inhibitor for lung cancer. On a theoretical basis we may presume that it may show additive effects with ARTs. However, effectiveness of sotorasib is limited to patients with p G12C KRAS mutation [159], which is not frequently found.
9. Reference [160] showed that ARTs were effective in pancreatic cancer cell killing *in vitro* and *in vivo*. This finding was further confirmed by [161].
10. Furthermore, [162] found that ARTs were able to induce ferroptosis in KRAS mutated pancreatic cancer cells. GRP78 (78 kDa glucose-regulated protein) was found to be an important inhibitor of artesunate-induced ferroptosis. GRP78 is frequently increased in PDAC [163] exerting pro-tumoral effects [164], [165] and chemoresistance [166]. Thus, inhibiting GRP78 represents an appropriate facilitator for pancreatic cancer treatment [167], [168]. Actually, it is the cell membrane GRP78 that needs to be targeted and there are many drugs that can bind or inhibit it, such as the BCR/ABL inhibitors imatinib and dasatinib, the antibiotic ceftriaxone, pemetrexed, sorafenib, zafirlukast, leucovorin, olaparib, among many others [169].
11. Triptolide, a natural compound consisting of three epoxy groups of diterpene lactone has shown potent anti/cancer effects in PDAC cells. When associated with artesunate the anticancer effects are synergistically increased [170]. Triptolide is excessively toxic and has been replaced by a more water soluble derivative minnelide for experimental purposes.
12. Ferroptosis is probably the main mechanism of the PDAC anti-cancer activity of artesunate, and probably this is in addition to apoptosis. Thus drugs that can induce ferroptosis can probably play an important role in the treatment of the disease. Reference [171] proposed two drugs to achieve ferroptosis: artesunate and zalsitabine (an anti HIV drug). The production of a considerable amount of reactive oxygen species in pancreatic cancer cells by artesunate, and leading to a non-apoptotic cell death has been confirmed by many authors [172], [173].
13. According to [174], artesunate downregulates pancreatic cancer cells growth and metastatic potential but at the same time increases the expression of angiogenic genes.

V. DISCUSSION

PDAC is still a wide-open problem. The main treatment approach – surgery – is only possible in less than 20% of patients. Furthermore, the tumor usually relapses within two years.

Non surgical cases have a very short survival in spite of chemotherapy. Gemcitabine as a stand-alone, or associated with nab-paclitaxel or cisplatin has not shown a real breakthrough regarding overall survival. The FOLFIRINOX scheme, although minimally more effective, introduced a slightly longer survival (in the range of weeks rather than months) at the price of high toxicity and multiple adverse events that negatively impact quality of life. However, gemcitabine remains the gold standard for this disease. It is evident that for longer and better-quality survival, gemcitabine needs to be "helped" somehow, beyond cisplatin and/or nab-paclitaxel. It is in this point where derivatives of artemisinin can play a role. Anti-malarial ARTs' diverse anti-cancer properties have been known for nearly 30 years ARTs benefits in cancer are tissue and tumor dependent to a certain

extent. In this review we examined the potential of ARTs complementing gemcitabine and increasing its effects in PDAC.

One of gemcitabine's problems is that not all the patients are responsive to the treatment and those who respond become resistant after a short time. ARTs can probably modify this situation.

For example, gemcitabine resistance in many cases is related to the activity of the MDR proteins that extrude the drug and/or decrease pro-apoptotic proteins. On the other hand, without fundamentally MDR proteins, ARTs, use a different mechanism to cause cell death: ferroptosis. This programmed cell death relies on an oxidative stress that induces lipid peroxidation and a high level of free oxygen radicals that unleash a caspase-independent cell death. Furthermore, no cross resistance was found between ARTs and standard chemotherapeutic drugs. Ferroptosis is the product of ARTs endoperoxide bridge reductive cleavage, reacting with an iron atom and forming free radicals [175]-[177]. Since cancer cells have a significantly higher intracellular iron content, they are more susceptible to ARTs cytotoxicity.

Fig. 2 explains the possible cooperation between gemcitabine and ARTs.

In 1998, [178] showed that an intracellular concentration of H_2O_2 of around $50 \mu M$ induced caspase-dependent apoptosis. Higher concentrations produced caspase-independent necrosis. Importantly, if the cells were treated with antioxidants, neither apoptosis nor necrosis occurred.

Reference [179] found gemcitabine to be an inducer of NADPH oxidase that increases ROS production, confirming previous reports in this regard. ROS production by gemcitabine has two undesirable effects:

1. It increases antioxidant synthesis [180] that prevents further apoptosis or necrosis;
2. It increases CXCR4 expression that induces migration and invasion [181].

While it is trying to annihilate the tumor, gemcitabine "is the cause of its own disgrace". Here Artesunate can help:

1. Artesunate impedes the production of antioxidants by inhibiting GPX4 (glutathione peroxidase 4) [182]; GPX4 is the lipid repair enzyme that corrects lipid peroxidation. Its inhibition creates lipid-based reactive oxygen species.
2. Induces an extreme redox stress that causes a programmed cell death different from apoptosis: ferroptosis.

Ribonucleotide reductase (RNR) is a key enzyme in DNA synthesis; by catalyzing the conversion of ribonucleotides into deoxyribonucleotides, it provides diphosphate deoxyribonucleotides as precursors for DNA. Therefore it is essential for tumor growth and proliferation [185]-[187]. One of the anti-cancer effects of gemcitabine is that it inhibits RNR [188], [189]. RNR over-expression or RNR resistance to gemcitabine is a major cause of gemcitabine-based treatment failure [190], [191]. Furthermore, inhibiting RNR results in increased sensitivity to gemcitabine and can overcome resistance in pancreatic cancer [192]. Importantly, artesunate is able to down-regulate RNR expression [155].

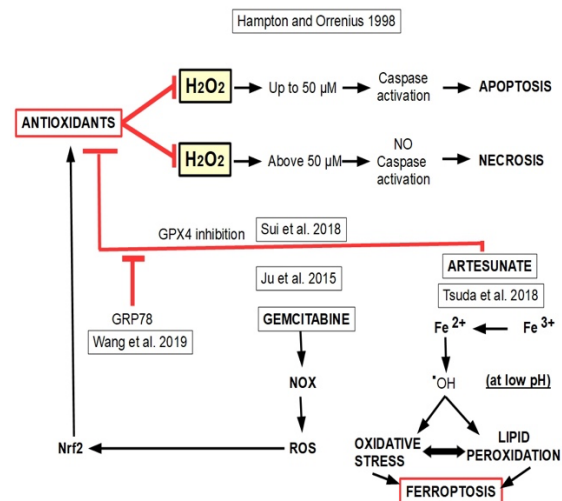


Fig.2. Artesunate is able to compensate two of gemcitabine's drawbacks: the compensatory production of antioxidants and bypass the ineffective apoptosis by ferroptosis. Blocking GRP78, artesunate's ferroptotic effects can be increased (not shown in the diagram). GRP78/BIP is a chaperone molecule of the endoplasmic reticulum that intervenes in protein quality control [183]. Iron dependence of lipid peroxidation in the diagram is based on reference [184].

Another usual mechanism of PDAC gemcitabine resistance is mediated through resistance to apoptosis [193]-[195]. ARTs are able to induce apoptosis but importantly it can kill malignant cells by a mechanism that is independent of and different from apoptosis: ferroptosis. This alternative cell death does not require the presence of apoptotic proteins but rather iron which is part of the redox deregulation created by ARTs.

Cytokines play a very important role in pancreatic cancer. All the pro-inflammatory cytokines active in acute pancreatitis are also found as a cause of the desmoplastic reaction in PDAC, namely tumor necrosis factor (TNF), platelet activating factor, IL-1, IL-6, IL-8, and IL-10 as the main players. ARTs were found to decrease many of these cytokines and inflammatory pathways [65], suppresses the IL-6/KAK/STAT signaling pathway [196], inhibits STAT3 [197], and decreases cytokine release from macrophages [198].

Artemisinin can disable the fibrotic process through the pathways mentioned and also by inhibiting other important mechanisms such as TGF- β , MAPK, Wnt/ β -catenin, PI3K/AKT, BMP-7 and Notch signaling [199]. Fibrosis, as part of the desmoplastic reaction in pancreatic cancer is an important cause of drug resistance because it impedes drug access to the tumor. ARS has shown to reduce fibrosis in the liver [200]. Interestingly, the same type of cells that cause hepatic fibrosis are operative in PDAC: stellate cells. In the liver, ARS inhibited proliferation and induced apoptosis of stellate cells [201], [202]. Although we lack experimental evidence to maintain that the same events may occur in PDAC, we do believe that this point deserves to be investigated. Evidence shows that ARS have anti-fibrotic effects in different tissues [203]. Desmoplastic reaction pathogenesis in PDAC is basically the same in different tissues, therefore, we may consider that it is not fundamentally different from what happens in other tumors and inflammatory desmoplastic responses in general.

There is overwhelming evidence of the anti-fibrotic effects of ARTs in liver and other tissues [204]-[206].

VI. CONCLUSIONS

ARTs have shown cytotoxic effects in multidrug resistant cells without cross-resistance. Thus, it can be considered an ultimate salvage drug when everything seems to be doomed to failure.

The areas of cooperation between gemcitabine and ARTs in pancreatic cancer can be summarized in the following points:

- 1) ARTs can increase sensitivity to gemcitabine by inhibiting RNR.
- 2) ARTs can reduce gemcitabine resistance by the same token.
- 3) ARTs are able to kill pancreatic cancer cells when apoptosis is decreased by using the ferroptotic pathway.
- 4) ARTs can eliminate the secondary production of anti-oxidants elicited by gemcitabine (Fig. 2).
- 5) ARTs can inhibit fibrosis thus reducing the desmoplastic reaction, which in turn increases gemcitabine's access to the tumor and impede the pro-tumoral crosstalk between stroma and tumor.
- 6) ARTs have independent cytotoxic effects that can add to gemcitabine cytotoxicity.
- 7) ARTs do not significantly increase chemotherapeutic side effects and exert no toxicity on normal cells.

Based on these parameters, we believe that time has come to test ARTs associated with gemcitabine in the first line treatment of PDAC.

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