Phytomedicine 22 (2015) 1045–1054

Contents lists available at ScienceDirect

Phytomedicine

journal homepage: www.elsevier.com/locate/phymed

Artemisinin derivatives induce iron-dependent cell death (ferroptosis) in tumor cells

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ARTICLE INFO

Article history:
Received 26 June 2015
Revised 3 August 2015
Accepted 5 August 2015

Keywords:
Cancer
Cell death
Iron
Microarray
Pharmacogenomics

ABSTRACT

Background: Apoptosis and other forms of cell death have been intensively investigated in the past years to explain the mode of action of synthetic anticancer drugs and natural products. Recently, a new form of cell death emerged, which was termed ferroptosis, because it depends on intracellular iron. Here, the role of genes involved in iron metabolism and homeostasis for the cytotoxicity of ten artemisinin derivatives have been systematically investigated.

Material and methods: $Log_{10}IC_{50}$ values of 10 artemisinin derivatives (artesunate, artemether, arteether, artenimol, artemisitene, arteanuin B, another monomeric artemisinin derivative and three artemisinin dimer molecules) were correlated to the microarray-based mRNA expression of 30 iron-related genes in 60 cell lines of the National Cancer Institute (NCI, USA) as determined in 218 different microarray hybridization experiments. The effect of desferoxamine and ferrostatin-1 on the cytotoxicity of artenimol of CCRF-CEM cells was determined by resazurin assays. The mRNA expression of *TFRC* was exemplarily validated by immunohistochemical detection of transferrin receptor protein expression.

Results: The mRNA expression of 20 genes represented by 59 different cDNA clones significantly correlated to the $log_{10}IC_{50}$ values for the artemisinins, including genes encoding transferrin (*TF*), transferrin receptors 1 and 2 (*TFRC, TFR2*), cerulopasmin (*CP*), lactoferrin (*LTF*) and others. The ferroptosis inhibitor ferrostatin-1 and the iron chelator deferoxamine led to a significantly reduced cytotoxicity of artenimol, indicating ferroptosis as cell death mode.

Conclusion: The numerous iron-related genes, whose expression correlated with the response to artemisinin derivatives speak in factor for the relevance of iron for the cytotoxic activity of these compounds. Treatment with ferroptosis-inducing agents such as artemisinin derivatives represents an attractive strategy for cancer therapy. Pre-therapeutic determination of iron-related genes may indicate tumor sensitivity to artemisinins. Ferroptosis induced by artemisinin-type drugs deserve further investigation for individualized tumor therapy.

Introduction

Artemisia annua L. (Asteraceae) has been used in traditional Chinese medicine for two millennia. In the second half of the 20th century, the antimalarial activity of artemisinin (**1**) from *A. annua* has been unraveled. This compound and its semisynthetic derivatives artemether (**2**) and artheether (**3**) are established drugs to combat infections with *Plasmodium falciparum* and *Plasmodium vivax* (Tu, 2011). As found by us and others in the 1990s, artemisinin derivatives also exert anticancer activity *in vitro* and *in vivo* (Efferth et al. 1996; Lai and Singh 1995). Interestingly, iron is a crucial determinant of activity of artemisinin-type drugs both in malaria and cancer. In both diseases, the activity of the drugs is associated with the presence of iron. This metal is present in large excess bound to hemoglobin in erythrocytes and fosters the cleavage of artemisinin's endoperoxide bridge in a Fenton-type chemical reaction. This leads to the generation of reactive oxygen species (ROS), which induce cell death of the *Plasmodia* parasites (Haynes et al., 2013). A comparable situation occurs in tumors. It is well known that the iron content is higher in tumors than in normal tissues explaining at least in part the

Abbreviations: DAB, diaminobenzidine; IC_{50}, 50% inhibition concentration; NCI, National Cancer Institute USA; ROS, reactive oxygen species; TFRC, transferrin receptor.

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http://dx.doi.org/10.1016/j.phymed.2015.08.002
0944-7113/

preferential cytotoxicity of artemisinins towards tumor cells compared to normal cells (Shterman et al. 1991). The exquisite susceptibility of tumor cells to artemisinins upon co-administration of holotransferrin or ferrous iron was reported by us and others (Lai and Singh 1995; Efferth et al. 2004; Kelter et al. 2007).

The best known mode of cell death is apoptosis, a programmed form of cell death occurring both in healthy and diseased cells (Cotter 2009). Two different pathways – an extrinsic receptor-driven and intrinsic mitochrondria-driven signaling cascade lead to the activation of caspases, which finally execute dying cells. In addition, other forms of cell death have been described. Autophagy, necroptosis, and mitoptosis may be induced instead or in parallel to apoptosis upon challenge of cells with toxic insults (Jain et al. 2013). The different forms of cell death have been intensively investigated for cytotoxic natural products (Efferth 2012; Lin and Tongyi 2014; Safarzadeh et al. 2014).

Recently, a novel type of caspase-independent non-apoptotic cell death has been described (Dixon and Stockwell 2014). It was termed ferroptosis, because it is dependent on the intracellular presence of iron. Ferric iron favors ROS generation and thereby induce of ferroptosis. It has been shown that RAS-mutated tumor cells commit programmed cell death with concomitant increases of ROS levels and decreases of mitochondrial sizes. The exact mechanism of ferroptosis has not been clarified yet. Intracellular cysteine import mediated by a glutamate-cysteine-antiporter systems in the cell membrane suppresses ferroptosis. Cysteine is needed for the synthesis if glutathione and glutathione prevents the accumulation of lipid peroxides. Ferroptosis may also occur by inhibition of glutathione peroxidase 4 (GPX4).

Erasin, an oncogenic RAS-selective lethal compound, as well as the kinase inhibitor sorafenib have been identified to inhibit the cysteine–glutamate antiporter complex x_c^- and to induce iron-dependent, oxidative cell death (Dixon et al. 2012, 2014). Ferrostatin-1 and deferoxamine are iron-depleting agents that inhibit ferroptosis (Louandre et al. 2013; Skouta et al. 2014).

Artemisinin exerts exquisite anticancer activity *in vitro* (Efferth et al. 1996, 2001, 2002, 2003b) and *in vivo* (Dell'Eva et al. 2004; Du et al. 2010; Li et al. 2007; Ma et al. 2011). Compassionate uses on single cancer patients as well as first clinical trials to treat veterinary and human tumors speak for the potential of artemisinin-type compounds as novel anticancer drugs (Berger et al. 2005; Breuer and Efferth 2014; Jansen et al. 2011; Krishna S 2014; Rutteman et al. 2013; Singh 2002; Zhang et al. 2008).

Artesunate (**5**) induced cell death in cancer cells by both the intrinsic and extrinsic pathways of apoptosis as well as autophagy and necroptosis (Button et al. 2014; Wang et al. 2012). In light of the multiplicity of cell death modes activated by artemisinin-type compounds, it is reasonable to hypothesize that they may also induce ferroptosis. There are already important hints that speak for this hypothesis. The role of holotransferrin and ferric iron in the form of iron (II)-glycine sulfate (Ferrosanol®) for the enhancement of tumor-inhibiting effects of artemisinins is well documented (Efferth et al. 2004; Kelter et al. 2007; Lai and Singh 1995).

However, the role of other proteins involved in iron metabolism and homeostasis for the activity of artemisinin-type drugs has not been addressed in detail as of yet. Therefore, genes encoding proteins involved in iron metabolism were systematically studied by a pharmacogenomic approach. To identify possible modes of action, the microarray-based mRNA expression of iron-related genes were correlated in the cell line panel of the National Cancer Institute (NCI), USA with the $\log_{10}IC_{50}$ values for 10 different artemisinin derivatives and found a large number of iron-related genes correlating with response to artemisinins. The names and chemical structures of the 10 artemisinin derivatives are shown in Fig. 1. Using the iron-depleting agents, deferoxamine and ferrostatin-1, the cytotoxic activity was diminished, which is another proof for ferroptosis as relevant cell death mode of artemisinin compounds.

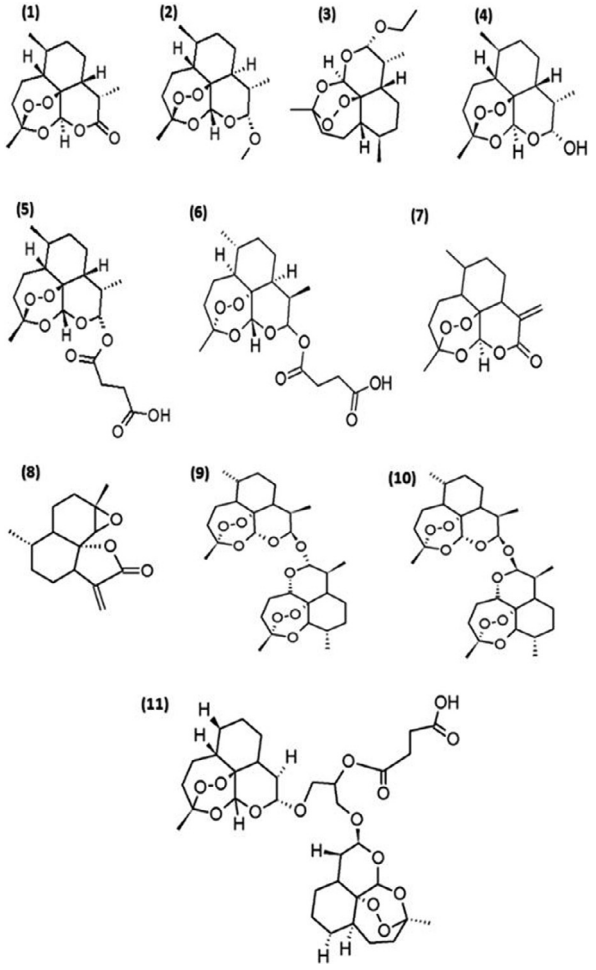

Fig. 1. Chemical structures of artemisinin and 10 derivatives.
(1)Trivial name: Artemisinin.
Systematic name: (1*R*,4*S*,5*R*,8*S*,9*R*,12*S*,13*R*)-1,5,9-trimethyl11,14,15,16-tetraoxatetracyclohexadecan-10-one. Mwt: 282.33218 g/mol.
(2)Trivial name: Artemether.
Systematic name: (1*R*,4*S*,5*R*,8*S*,9*R*,10*S*,12*R*,13*R*)-10-methoxy-1,5,9-trimethyl-11,14,15,16-tetraoxatetracyclohexadecane. Mwt: 298.375 g/mol.
(3) Trivial name: Arteether.
Systematic name: (3*R*,5a*S*,6*R*,8a*S*,9*R*,10*S*,12*R*,12a*R*)-10-ethoxydecahydro-3,6,9-trimethyl-3,12-epoxy-12H-pyrano(4,3-j)-1,2-benzodioxepin. Mwt: 312.40122 g/mol.
(4) Trivial name: Artenimol.
Systematic name: (3*R*,5a*S*,6*R*,8a*S*,9*R*,12*R*,12a*R*)-3,6,9-trimethyldecahydro-3,12-epoxypyrano[4,3-j][1,2]benzodioxepin-10-ol. Mwt: 284.34806 g/mol.
(5) Trivial name: Artesunate.
Systematic name: 4-oxo-4-{[(4*S*,5*R*,8*S*,9*R*,10*S*,12*R*,13*R*)-1,5,9-trimethyl-11,14,15,16-tetraoxatetracyclohexadec-10-yl]oxy}butanoic acid. Mwt: 384.42082 g/mol.
(6) Trivial name: Artesunate derivative.
Systematic name: 4-oxo-4-{[(3*R*,5a*S*,6*R*,8a*S*,9*R*,10*R*,12*R*,12a*R*)-3,6,9-trimethyldecahydro-3,12-epoxy[1,2]dioxepino[4,3-i]isochromen-10-yl]oxy}butanoic acid. Mwt: 384.4208 g/mol.
(7) Trivial name: Artemisetene.
Systematic name: (12*S*)-3,6-dimethyl-9-methylideneoctahydro-3,12-epoxy[1,2]dioxepino[4,3-i]isochromen-10(3h)-one. Mwt: 280.3163 g/mol.
(8) Trivial name: Arteanuine B.
Systematic name: (1b*R*,7*R*,9a*R*)-7,9a-dimethyl-4-methylenedecahydro-3H-oxireno[7,8]naphtho[8a,1-b]furan-3-one. Mwt: 248.3175 g/mol.
(9) Trivial name: Artemisinin dimer 1 (stereoisomer of 10).
Systematic name: 3,12-epoxy-12H-pyrano[4,3-j]-1,2-benzodioxepin, 10,10′-(oxy)bis[decahydro-3,6,9-trimethyl-,stereoisomer. Mwt: 566.68024 g/mol.
(10) Trivial name: Artemisinin dimer 2 (stereoisomer of 9).
Systematic name: 3,12-epoxy-12H-pyrano[4,3-j]-1,2-benzodioxepin, 10,10′-(oxy)bis[decahydro-3,6,9-trimethyl-,stereoisomer. Mwt: 566.68024 g/mol.
(11) Trivial name: Artemisinin dimer hemisuccinate.
Systematic name: 3,12-epoxy-12H-pyrano[4,3-j]-1,2-benzodioxepin, 10,10′-[(2-hydroxy-1,3-propanediyl)bis(oxy)] bis[decahydro- 3,6,9-trimethyl-, hemisuccinate. Mwt: 724.83214 g/mol.