RESEARCH COMMUNICATION

Cytotoxic Activity of Artemisinin Derivatives Against Cholangiocarcinoma (CL-6) and Hepatocarcinoma (Hep-G2) Cell Lines

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Abstract

Cytotoxic activity of artemisinin and derivatives in the presence and absence of holo-transferrin and expression of genes involved in resistance of cancer cells were investigated in human cholangiocarcinoma (CL-6) and hepatocarcinoma (Hep-G2) cell lines in vitro. After incubation with the test drugs and 5-fluorouracil (5-FU) cytotoxicity was asessed by MTT assay. RNA was extracted after 24 hour exposure to holo-transferrin for invesstigation of the expression of transferrin receptor 1 (TDR1), multidrug resistance 1 (MDR1), multidrug resistance protein 1 (MRP1), multidrug resistance protein 2 (MRP2), and multidrug resistance protein 3 (MRP3). The median IC_{50} of artemisinin, artesunate, β-artemeter, dihydroartemisinin and 5-FU were as follows: CL-6: 339, 131, 354, 75, and 377 μM, respectively; Hep-G2: 268, 50, 233, 29, and 1,380 μM. Exposure to holo-transferrin had no influence on sensitivity of either cell line to artemisinin derivatives, but resulted in a 3-fold increase in the expression of TR1 and MDR1, and a 2-fold increase in the expression of MRP1 and MRP2 in CL-6 cells. With Hep-G2, a 3-fold increase in the expression of MDR1 and MRP3 and a 2-fold increase in expression of MRP2 were observed. Dihydroartemisinin exhibited the most potent cytotoxic activity against both cell lines and holo-transferrin caused different patterns of expression of resistance-associated genes.

Keywords: Artemisinin - cholangiocarcinoma - cytotoxicity - transferrin

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Introduction

Artemisinin is a chemical compound extracted from the wormwood plant, *Artemisia annua L.* Its chemical structure is a sequiterpene trioxane lactone with an endoperoxide bridge that is essential for its activity against malaria parasites. The endoperoxide interacts with a Fe (II) to form free radicals (Rosenthal and Meshnick, 1996; Dhingra et al., 2000). Due to a high amount of Fe (II) in the form of heme molecules in malaria parasite, artemisinin's antimalarial bioactivity is interacted with the intraparasitic iron source and generation of free radical leading to cellular destruction (Meshnick, 2002). Recently, artemisinin and derivatives (artesunate, β-artemeter and dihydroartemisinin) have been shown to exert cytotoxic activity against various cancer cells, e.g., melanoma, breast, ovarian, prostate, CNS and renal cancers (Chadwick et al., 2009; Chen et al., 2009a; 2009b; Nakase et al., 2009). Cancer cells were mainly by inducing apoptosis, while normal cells were essentially non-toxic (Nakase et al., 2008). Moreover, artemisinin and derivatives were shown to inhibit the growth of fibrosarcoma, breast and ovarian cancers in rat model (Moore et al., 1995; Chen et al., 2004; Lai and Singh 2006).

High rate of iron intake has been observed in most cancer cells (Karin and Mintz, 1981; Shterman et al., 1991). In addition, the cells also express high cell surface concentration of transferrin receptors (Reizenstein, 1991; Raaf et al., 1993), which enhances the binding of iron to the iron binding protein transferrin (iron-bound transferrin or holo-transferrin). Iron enters into the cells via a receptor-mediated endocytosis process (May and Cuatrecasas, 1985). Therefore, the iron storage of tumor cells is generally greater in tumor than in normal cells (Shterman et al., 1991). Based on this observation and due to the fact that artemisinin derivatives target heme-iron complex, they would be expected to enhance toxicity of the pre-loading tumor cells with iron. Holo-transferrin and other iron sources have clearly been shown to increase the potency of artemisinin in killing cancer cells (Singh and Lai, 2001; Sadava et al., 2002; Lai and Singh, 2006; Nakase et al., 2009).

In the present study, the cytotoxic activity of artemisinin and derivatives (artesunate, β-artemeter and dihydroartemisinin) in the presence and absence of holo-transferrin were investigated against human cholangiocarcinoma (CL-6) and hepatocacinoma (Hep-G2) cell lines in vitro. In addition, the inducing effect

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of these compounds on the expression of genes involved in resistance of cancer cells to chemotherapeutics were also investigated.

Materials and Methods

Cell lines

The human cholangiocarcinoma (CL-6) and hepatocarcinoma (Hep-G2) cell lines were used in the study. CL-6 cell line was established and kindly provided by Associate Professor Dr. Adisak Wongkajornsilp, Department of Pharmacology, Faculty of Medicine (Siriraj Hospital), Mahidol University, and was maintained in culture at the Pharmacology and Toxicology Unit, Graduate Program in Biomedical Sciences, Thammasat University, in Ham's F12 medium containing 100 U/mL penicillin and 100 μg/mL streptomycin with 10% fetal bovine serum. Hep-G2 cell line was purchased from the Cell Line Service Co. Ltd. (Germany) and was maintained in DMEM: Ham's F12 medium supplemented with 2 mM L-glutamine, 10% fetal bovine serum and 100 IU/ml pen-strep. All cell lines were maintained in an atmosphere of 50 mL/L CO_2 at 37°C.

Drug solutions

Standard powder of artemisinin, artesunate, β-artemeter and dihydroartemisinin were kindly provided by Dafra Pharma, Belgium. 5-Fluorouracil (5-FU) and holo-transferrin were purchased from MP Biomedicals (France) and Sigma (USA), respectively. Stock solutions of all drugs were prepared at the concentration of 10 mM in 50% ethanol. Holo-transferrin was prepared at a stock concentration of 12 μM in sterile water, and diluted to obtain final concentration of 1 μM. A series of serial dilutions of each drug was prepared to allow for the determination of IC_{50} value (drug concentration which produces 50% cell growth inhibition) of the individual drug. The final concentrations of artemisinin, artesunate, β-artemeter and dihydroartemisinin were 1000, 500, 250, 125, 62.5, 31.25, 15.625 and 7.8125 μM, whereas the final concentrations for 5-FU were 2000, 1000, 500, 250, 125, 62.5, 31.25 and 15.625 μM.

Cytotoxicity assay

The sensitivity of the two cell lines to artemisinin, artesunate, dihydroartemisinin, ß-artemter and 5-FU, in the presence and absence of holo-transferrin was determined by MTT assay with modification (Futaki et al., 2004). Briefly, cell suspension at density of 1×10^4 cells/well was added to each well of 96-well flat-bottomed microtiter plate and the plate was incubated at 37°C for 24 h in a humidified 50 mL/L CO_2 atmosphere. After incubation, medium with or without 1 μM of holo-transferrin was added in each well and the plate was further incubated at 37°C for 4 h. Culture medium was thereafter carefully removed, and the medium containing test drugs or complete medium (untreated control) was distributed in each well. Following an incubation at 37°C for an additional 48 h, 10 μl of MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] solution (5 mg/mL) was added to each culture well. The culture was further incubated at 37°C for an additional 2 h in a humidified 50 mL/L CO_2 atmosphere. Absorbance was measured at 570 nm using a microplate reader (Varioscan Flash, Thermo, Finland). Concentration-response analysis was performed using Calcusyn™ version 1.1 (Biosoft Software, UK) to calculate the IC_{50} values. Three separate experiments, triplicate each, were performed for each drug against each cell line. Relative potency of each test drug was determined from the ratio of median IC_{50} value of test drug and 5-FU.

Expression of resistance genes following holo-transferrin exposure

Expression of transferrin receptor 1 (TDR1) and the genes that would be involved in resistance of cancer cells to chemotherapeutics (multidrug resistance 1: MDR1, multidrug resistance protein 1: MRP1, multidrug resistance protein 2: MRP2; and multidrug resistance protein 3: MRP3) was investigated in the presence (4 and 24 hour exposure) and absence of exposure of CL-6 and Hep-G2 cell lines to holo-transferrin. RNA extraction was extracted from both cell lines by using RNeasy mini Kit (QIAGEN). cDNA was synthesized by Superscript™ III First-Strand Synthesis (Invitrogen, USA). Gene expression was determined by SYBR Green I real-time PCR (iCycler, Bio-Rad, USA) using the default thermocycler program for all genes: 10 minutes of pre-incubation at 95°C followed by 40 cycles for 15 seconds at 95°C and one minute at 60°C. The oligonucleotide primers used are shown in Table 1. Individual real-time PCR reaction was carried out in 25 μl volume in a 96-well plate containing 1× buffer (10×), 3.5 mM $MgCl_2$, 200 μM dNTPs, 1 μM of sense and antisense primers and 12.5 μl of PlatinumTM PCR SuperMix (Invitrogen, USA).

Results were analyzed by a comparative Ct method based on the assumption that the target (TR1, MDR1, MRP1, MRP2 and MRP3) and reference (Glyceraldehyde-3- phosphate) genes were amplified with the same efficiency within an appropriate range of cDNA concentrations. The ΔΔCt calculation for the relative quantification of target was as follow: ΔΔCt = (Ct, target gene – Ct, GAPDH)χ – (Ct, target gene – Ct, GAPDH)y, where χ is sample and y is cell line without holo-trnasferrin (baseline). Result for each sample was

Table 1. Primer Sequences for Investigation of Gene Expression

Primer Name	Sequence (5'-3')
TR1-F	CTGCACCAGGCTCTATCCTAG
TR1-R	GTACCTAACTCTGCACAGGTG
GAPDH-F	CAACAGCCTCAAGATCATCAGC
GAPDH-R	TTCTAGACGGCAGGTCAGGTC
MDR1-F	GTCTTTGGTGCCATGGCCGT
MDR1-R	ATGTCCGGTCGGGTGGGATA
MRP1-F	CTGACAAGCTAGACCATGAATGT
MRP1-R	CCTTTGTCCAAGACGATCACCC
MRP2-F	GCCAGATTGGCCCAGCAAA
MRP2-R	AATCTGACCACCGGCAGCCT
MRP3-F	GGGACCCTGCGCATGAACCTG
MRP3-R	TAGGCAAGTCCAGCATCTCTGG

TR, transferrin; GAPDH, glyceraldehyde-3-phosphate; MDR, multidrug-resistance; MRP, multidrug-resistance protein

Table 2. The Median IC_{50} (Range) and Relative Potency Values of Artemisinin, Artesunate, β-artemter, Dihydroartemisinin and 5-FU Against CL-6, Hep-G2 and HEp-2 Cell Lines, in the Presence and Absence of Holo-transferrin

Drug	Median IC_{50} (range: μM) and (relative potency)	
	CL-6	Hep-G2
Artemisinin	339 (251-427) (1.1)	268 (167-369) (1.4)
Artemisinin + holo-transferrin	458 (292-624) (0.8)	258 (148-368) (1.5)
Artesunate	131 (68-194) (2.9)	50 (41-59) (7.6)
Artesunate + holo-transferrin	112 (66-158) (3.4)	46 (45-47) (8.3)
β-Artemeter	354 (196-512) (1.1)	233 (55-411) (1.6)
β-Artemeter + holo-transferrin	294 (199-389) (1.3)	260 (63-457) (1.5)
Dihydroartemisinin	75 (57-93) (5.0)	29 (5-53) (13.0)
Dihydroartemisinin + holo-transferrin	75 (64-86) (5.0)	32 (14-50) (12.0)
5-FU	377 (222-532) (1.0)	380 (217-543) (1.0)

* Relative potency: the ratio of median IC_{50} value of test drug and 5-FU

expressed as an N-fold change in χ target gene expression normalized to GAPDH, relative to the gene expression in cell line with non-exposure to holo-trnasferrin as follow: amount of target = $2^{-\Delta\Delta Ct}$. A minimum of two experiments was carried out for each gene and each sample. In each experiment, each individual sample was run in duplicate wells and the Ct of each well was recorded at the end of the reaction.

Statistical analysis

The median (range) of IC_{50} including relative potency values for all drugs against the two cell lines, in the presence or absence of holo-transferrin were compared using Mann-Whitney U-test for data not conforming to normal distribution (SPSS version 12). Statistical significance level was set at $\alpha = 0.05$.

Results

Cytotoxic effect of artemisinin and derivatives in the presence and absence of holo-transferrin exposure

Cytotoxic effects of artemisinin derivatives in the presence and absence of holo-transferrin exposure were similar in both cell lines. Both cell lines were markedly less sensitive to 5-FU compared with artemisinin derivatives, with IC_{50} values ranging from 377-380 μM (Table 2). In the absence of holo-transfferin exposure, the median IC_{50} (range) values of artemisinin, artesunate, β-artemeter, dihydroartemisinin and 5-FU for CL-6 and Hep-G2 cell lines were as follows: CL-6: 339 (251-427), 131 (68-194'), 354 (196-512), 75 (57-93) and 377 (222-532) μM; Hep-G2: 268 (167-369), 50 (41-59), 233 (55-411), 29 (5-53) and 380 (217-543) μM. The order of relative potencies for cytotoxic activities of artemisinin and derivatives against CL-6 and Hep-G2 were: dihydroartemisinin > artesunate > β-artemeter > artemisinin (Table 2). The relative potencies of the most potent derivative dihydroartemisinin against Hep-G2 and CL6 cell lines were 13.0, and 5.0, respectively. The activities of artemisinin derivatives against CL-6 was about 3-4 fold lower than Hep-G2 cell line. In the presence of holo-transferrin exposure, the median (range) values of artemisinin, artesunate, β-artemter and dihydroartemisinin for CL-6 and Hep-G2 cell lines were: CL-6: 458 (292-624), 112 (66-158), 294 (199-389) and 75 (64-86) μM; Hep-G2; 258 (148-368), 46 (45-47), 260 (63-457) and 32 (14-50) μM (Table 2).

Table 3. Expression of TR1, MDR1, MRP1, MRP2 and MRP3 Genes When Exposed to Holo-transferrin; Data are Expressed as Baseline Comparison 91 Gene Copy Number)

	Fold- change of gene expression				
	TR1	MDR1	MRP1	MRP2	MRP3
CL-6-0h (baseline)	1	1	1	1	1
CL-6-4h	2	2	2	2	1
CL-6-24h	3	3	2	2	1
HepG2-0h (base line)	1	1	1	1	1
HepG2-4h	1	1	1	2	2
HepG2-24h	1	3	1	2	3

Expression of resistance genes following exposure to holo-transferrin

For CL-6 cell lines following a 24-hour exposure to holo-transfeerin, a 3-fold increase in the expression of TR1 and MDR1 and a 2-fold increase in the expression of MRP1 and MRP2 were observed. There was no change in the expression of MRP3 gene. For Hep-G2 cell lines, a 3-fold increase in the expression of MDR1 and MRP3 and a 2-fold increase in the expression of MRP2 were observed. No change in the expression of TDR1 and MRP1 genes was found.

Discussion

The present study investigated in vitro cytotoxic activity of artemisinin and derivatives against CL-6 and Hep-G2 cell lines in comparison with the standard anticancer drug 5-FU. The relatively high IC_{50} value of 5-FU observed with CL-6 cell line is in agreement with what was previously reported in cholangiocarcinoma cell lines obtained from Thai patients, where its mean IC_{50} values against KKU-100 and KKU-M156 cell lines were 1,018 and 144 μM, respectively (Tepsiri et al., 2005). The IC_{50} value of 5-FU in cholangiocarcinoma cell lines is considered markedly high when compared with other cancer cell lines including colon carcinoma -- HCC-48 and COLO20 cell lines (8.6 and 16 μM, respectively)

(Nishiyama et al., 1999) and cervical squamous carcinoma -- SiHa and HeLa cell lines (11.4 and 2.8 μM, respectively) (Iida et al., 2001; Laochariyakul et al., 2003). The IC_{50} values of artemisinin against CL-6 and Hep-G2 observed in the present study were similar (>50 μM) (Michaelis et al., 2010).

Cytotoxic activity of artemisinin and derivatives against CL-6 was noticeably varied (median IC_{50} values 75-339 μM), with dihydroartemisinin being the most potent compound. Similarly to 5-FU, the IC_{50} values of artemisinin and derivatives were also remarkably high in this type of cancer cell when compared with those previously reported in human small-cell lung carcinoma -- H69 and H69VP cell lines (IC_{50} of artemisinin: 24 and 2.3 nM, respectively) (Sadava et al., 2002), leukemia cell line – CCRF-CEM: (IC_{50} of artemisinin and artesunate: 1.1 and 11.5 μM, respectively), astrocytes -- U373 cell line (IC_{50} of artemisinin and artesunate: 3.5 and 3.3 μM, respectively) (Efferth et al., 2004), prostate carcinoma -- DU 145 cell line (IC_{50} of dihydroartemisinin: 9.0 μM), Hep-G2 (IC_{50} of dihydroartemisinin: 29-258 μM) (Nakase Lai, 2008), and 16-neuroblastoma cell lines (IC_{50} of dihydroartemisinin and artesunate: 2.1-12.0 and 1.4-30.0 μM, respectively) (Michaelis et al., 2010). All these data may suggest that cholangiocarcinoma is more (innate) resistant to chemotherapeutic agents than other cancers. In addition, results also showed varying activity of artemisinin derivatives against these cell lines. Further study for clarification of the difference in potencies of artemisinin derivatives against these types of cancer particularly cholangiocarcinoma is required. One possible explanation could be difference in their physicochemical properties (Ploypradith, 2004; Golenser et al., 2006). Artesunate is soluble in water (Barradell and Fitton, 1995), while ß-artemether is soluble in oil (Brossi et al., 1988). The solubility of artemisinin is low in both water or oil (Hofheinz et al., 1994). Dihydroartemisinin exhibited the most potent cytotoxic activity against all cell lines and therefore, should be considered as the candidate derivative for chemotherapy of these cancers particularly cholangiocarcinoma. Nevertheless, considering its IC_{50} value in cancer cells of approximately 40,000 fold of that in K1 *Plasmodium falciparum* clone (IC_{50} = 1.7 nM), this raises concern over the toxicity of artemisinin derivatives in clinical use. Neurotoxicity of high doses artemisinin derivatives given by intramuscular injection have been reported in dogs (arteether at the dose of 20 mg/day for 8 days), and rats (artemether at the dose of 12.5 to 50 mg/kg/day for 28 days) (Brewer et al., 1994). On the other hand, oral and subcutaneous administration of the new artemisinin derivative RO42-1611 (arteflene) at 400 mg/kg/day for 4 weeks was well tolerated and did not induce any mutagenic effect in rat (Hofheinz et al., 1994). To obtain optimal dosage regimens, pharmacokinetic (concentrations of drug in plasma and cancer cells)/pharmacodynamic (cytotoxic activity and patient's tolerability) relationship of artemisinin derivatives in cancer chemotherapy needs to be investigated. Therapeutic dose of artemisinin derivatives for the treatment of cholangiocarcinoma is expected to be much higher than that used for treatment of malaria. In patients with acute uncomplicated *P.falciparum* malaria, artemisinin derivatives are well tolerated when used as monotherapeutic agents in the dose range of 2-6 mg/kg body weight given once every day for 5-7 days (Gordi and Lepist, 2004).

It is noteworthy that pretreatment of CL-6 and Hep-G2 cell lines with holo-transferrin did not alter the cytotoxicity of artemisinin and its derivatives. The possible explanation is the up-regulation of multidrug resistance genes in cancer cells after exposure to holo-transferrin. The expression of TR1 in CL-6 after exposure to holo-transferrin was increased; yet, other resistance genes were also concurrently increased. These may explain the lack of effect of holo-transferrin on the activity of artemisinin derivatives in CL-6 cell line. It is noted however for the unchanged in expression of TR1, but other genes in Hep-G2 cell line.

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