

Antitumor Activity of the Combination of Artemisinin and Epirubicin in Human Leukemia Cells

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Received: 29 June 2020 ♦ **Accepted:** 30 July 2020 ♦ **Published:** 31 Aug 2021

Citation: Zhelyazkova MY, Hristova-Avakumova NG, Momekov GTsv. Antitumor activity of the combination of artemisinin and epirubicin in human leukemia cells. *Folia Med (Plovdiv)* 2021;63(4):488-95. doi: 10.3897/folmed.63.e55938.

Abstract

Aim: We evaluated the tumor-inhibiting effect of artemisinin applied separately and in combination with epirubicin on leukemia HL-60 and HL-60/Dox cell lines, its dose modulation effect and its potency to influence iron-induced oxidative damage of biologically relevant molecules.

Materials and methods: MTT assay and the method of Chou-Talalay were used to show the inhibition of tumor cell proliferation and to evaluate the synergistic effect and modulation effect of artemisinin and epirubicin at varying concentrations. We also used spectrophotometric assays to determine the potency of artemisinin to influence iron-induced molecular degradation of lecithin and deoxyribose.

Results: Artemisinin exhibits tumor-inhibiting effect on both the anthracycline-sensitive and anthracycline-resistant promyelocytic cell lines, reaching 88% and 61% (T/C), respectively, when applied at higher concentrations in a dose-dependent manner. The combination of artemisinin and epirubicin shows synergistic effects in all tested concentrations on doxorubicin-resistant cells (CI<0.7). Artemisinin sensitizes the resistant cells towards epirubicin as shown by the CI (combination index) values and has a dose-modulation effect as shown by DRI (dose reduction index). Artemisinin induces deoxyribose oxidative degradation when applied alone and exerts synergistic deoxyribose degradation effect when applied with iron. However, artemisinin does not influence the studied processes in the lecithin-containing model system and has no potential to induce lipid peroxidation.

Conclusions: This study presents a new opportunity to enhance the effectiveness of epirubicin-based treatment regimens with addition of artemisinins for resistant tumors.

Keywords

combination chemotherapy, deoxyribose, HL-60/Dox, lecithin, synergism

INTRODUCTION

Resistant tumors are subject to many investigations in the field of combination chemotherapy. Epirubicin (EPI), an anthracycline antibiotic, is widely used for the treat-

ment of solid tumors. Despite the remarkable anticancer activity of EPI, its high-dose therapeutic use is limited by cardiotoxicity and secondary drug resistance.¹ Therefore, its inclusion in combination treatment may be highly desirable.

Traditional medicinal herbs are widely used as an attractive source of therapeutic regimens without a severe toxicity. So is *Artemisia annua* L., belonging to the plant family of *Asteraceae* and containing the biologically active sesquiterpene lactone artemisinin (ART), with a unique 1,2,4-trioxane ring that causes free radical-induced damage. For many years, ART has been effectively used for the treatment of drug-resistant malaria. Today, this drug and its semisynthetic derivatives, named artemisinins, are produced thanks to a modern biotechnological approach.² They possess antimalarial, antitumor, and antiviral activity, block the angiogenesis and inhibit metastases.³⁻⁶ Also, they induce apoptosis and ferroptosis by producing carbon-centered radicals following bioactivation with iron.⁷⁻¹¹ Thus, they are a typical example of bioreductive drugs, such as mitomycin, etoposide, and some quinone-containing agents.¹² In addition, lysosome-induced autophagy has been described as another mechanism of action.^{4,13}

Some of the artemisinins such as dihydroartemisinin, artesunate, and artemisone enhance the antitumor potential of oxaliplatin, carboplatin, gemcitabine or doxorubicin.^{5,9,14} But data for ART remains contradictory and limited: there is evidence for additive effect in combination with thalidomide, and antagonism in combination with oxaliplatin and gemcitabine on colon and mammary tumor cells.^{15,16} Some authors hypothesized that the combination of artemisinins with cytostatics may improve their antitumor activity on resistant cells due to chemosensitization and radical-mediated cytotoxicity.¹⁷

AIM

In this study we evaluated the effectiveness of epirubicin-based combinations with artemisinin on sensitive HL-60 and resistant to anthracyclines HL-60/Dox cell lines, its dose modulation effect and its potency to influence iron-induced oxidative damage of biologically relevant molecules.

MATERIALS AND METHODS

Reagents, reactives and drugs

The chemicals used in the spectrophotometric assays were obtained from Sigma-Aldrich and are presented under the correlating figures; artemisinin, caps. 100 mg from Science-Based Nutrition, USA; epirubicin (Farmorubicin, fl 2 mg/ml) from Pfizer; MTT (3-(4,5-dimethylazol-2-yl)-2,5-diphenyltetrazolium bromide) from Genaxxon.

Cell lines and culture

HL-60 and HL-60/Dox promyelocytic leukemia cell lines

were obtained from DSMZ (Braunschweig, Germany). The cells were maintained at 37°C under an atmosphere of 5% CO₂ in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, L-glutamine (final concentration 2.5 mM) and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin).

MTT assay

A slightly modified version of MTT assay was performed to determine the cell survival fraction.¹⁸ Exponentially growing HL-60 and HL-60/Dox cells were seeded into 96-well plates. The cells were treated with the indicated drugs in 3-5 different concentrations, and the cell viability was determined after 72 hours of incubation, adding MTT (10 mg/ml). Absorption was measured by an automated microtiter plate spectrophotometer (Labexim LMR-1, Lengau, Austria) at 550 nm.

Method of Chou-Talalay^{19,20}

We applied the mathematical algorithm of Chou to assess the effectiveness of the combination [ART+EPI] in varying and fixed-ratio concentrations based on the median effect (Fa) of the two drugs applied separately and simultaneously derived from the cell vitality assay. According to this method, the combination index (CI) value for each concentration scheme less than 0.9 denotes synergism, CI=0.9-1.10 denotes additive interaction, and CI more than 1.10 denotes antagonism; the dose-reduction index (DRI) equal to one denotes no dose reduction, whereas DRI>1 and DRI<1 indicates favourable and unfavourable dose-reduction, respectively. In addition, we analyzed bioequivalent concentrations (IC₅₀) for some of the schemes.

Iron-induced oxidative molecular damage

The assay was performed by using a modification of the ferrous iron method described by Asakawa and Matsushita.²¹ The samples were prepared in phosphate buffer (K₂HPO₄/KH₂PO₄, pH 7.4) with equivalent concentrations of the oxidizable substrate lecithin (1 mg/ml) or deoxyribose (0.5 mmol/L). The peroxidation was induced by a typically used agent FeCl₂ (0.1 mmol/L). A sample named Control containing the biologically relevant molecule and Fe²⁺, was prepared where ART had been omitted. The effect of the compound alone at the maximal tested concentration under the used experimental conditions was also determined. All samples were incubated at 37°C for 30 min. Then 0.5 ml of 2.8% trichloroacetic acid and 0.5 ml of thiobarbituric acid were added. The mixtures were heated in a boiling water bath at 100°C for 20 min. The test tubes were cooled at room temperature and then centrifuged at 3000 rpm for 20 min. The absorbance at 532 nm was measured. The observed oxidative damage named “molecular damage” was presented as a percentage of the control sample treated only with Fe²⁺.

Statistical analysis

The computer software CalcuSyn (Biosoft, UK) was used to define different parameters: median effect (Fa), combination index (CI), dose reduction index (DRI) and bioequivalent concentrations, and to assess quantitatively the combination drug effect. The experimental data was reported as mean \pm SD of at least three different experiments (n=3). A student t-test was used to determine the difference between the two groups. All the graphs were compiled and statistical analyses were performed using GraphPad Prism.8, MS Excel and Origin Plot.

RESULTS

Tumor-inhibiting effect

We first examined the effects of ART and EPI alone on the proliferation of HL-60 and HL-60/Dox cells (**Figs 1A, 1B**). The MTT assay showed that ART has antitumor activity on HL-60 leukemia cells (68% and 88% T/C is reached at 400 and 800 $\mu\text{mol/L}$, respectively). Almost the same tumor inhibition was achieved (61% - at 400 $\mu\text{mol/L}$) on the resistant HL-60/Dox cells. At lower concentrations (100 and 200 $\mu\text{mol/L}$) ART did not show activity over the desired criteria of 50% and many of the sensitive and resistant cells remained vital. These results show that ART does not have cross-linked resistance with the anthracyclines.

EPI applied separately eliminated approximately all vital HL-60 cells at concentrations of 2.5 and 5 $\mu\text{mol/L}$ but not at 1.25 $\mu\text{mol/L}$. After the combined treatment, markedly stronger anti-proliferation activity was achieved when 200 $\mu\text{mol/L}$ ART was added to 1.25 $\mu\text{mol/L}$ EPI: the inhibiting effect of the combination was evaluated as 98%, and that is two times more than that of EPI alone. In the other cases it is impossible to analyze comparatively the effectiveness of the combination treatment because EPI alone eliminates all leukemia cells. In contrast, EPI inhibits only less than 10% in all tested concentrations when applied separately on

the resistant HL-60-Dox cells. But co-treatment with ART diminished vitality of the tumor cells in a dose-dependent manner. The tested combination scheme [5 EPI + 400 ART, $\mu\text{mol/L}$] reached a maximal inhibiting effect of 92%. This effect is higher than the one achieved by ART alone, and the same as the dose-response of the sensitive leukemia cells to EPI.

Quantitative assay by CI

We assessed the effectiveness of varying and fixed-ratio concentrations of [epirubicin+artemisinin]. Synergy on the sensitive HL-60 cells was obtained in varying concentrations only when ART was in the lowest tested concentration of 200 $\mu\text{mol/L}$ (CI<0.9) (**Table 1A**). In the combination [1.25 EPI + 400 ART, $\mu\text{mol/L}$] the observed effect was additive (0.9<CI<1.10). These effects were confirmed by the results from the estimated fixed-ratio concentration of EPI:ART equal to 1:160. The highest tested concentrations of the two drugs, applied simultaneously on HL-60, had CI values higher than one, denoting antagonism. But, as it is shown in **Fig. 1A**, this is due to the absence of vital cells after exposure with EPI. Only the scheme [1.25 EPI + 200 ART, $\mu\text{mol/L}$] had CI=0.47, denoting significant synergism.

The assay of the dose-response on the resistant HL-60/Dox for varying concentrations of ART and EPI showed higher effectiveness in all of the tested dosage regimens (**Table 1B**). These CI values denote synergism and potentiation of the tumor inhibition after combination treatment. The same results were obtained for the fixed-ratio concentrations of EPI:ART equal to 1:80 (all values of CI<0.9).

Dose modulation effect and DRI

The dose modulation effect of ART and EPI was evaluated by means of DRI for the fixed ratio concentration on the basis of computer-simulated dose-effect data (**Table 2**). It was observed that in all cases the concentration of EPI may be diminished according to the indicated concentrations of ART (DRI>1) and the effect (Fa) is conserved at all levels (25% to 90% inhibition). Favourable reduction of

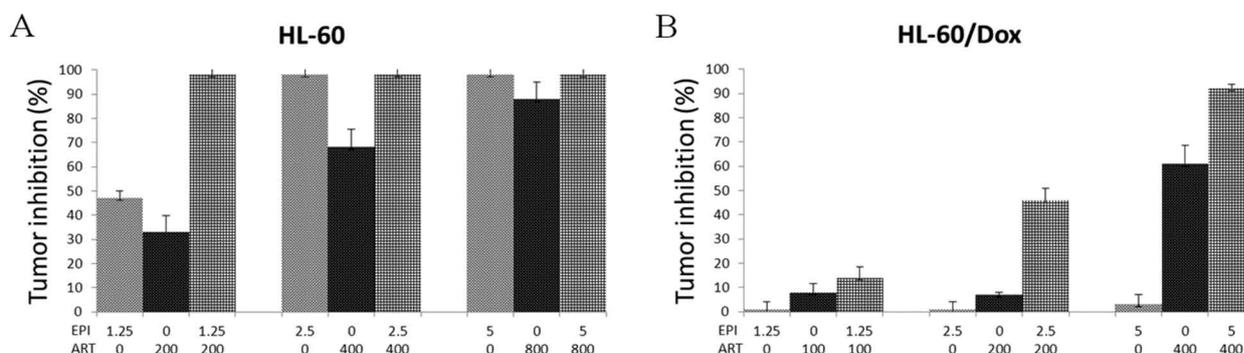


Figure 1. Cytotoxicity of [artemisinin+epirubicin] in leukemia cell lines. **A.** Dose-response of HL-60 to epirubicin, artemisinin and [artemisinin+epirubicin]. **B.** Dose-response of HL-60/Dox to epirubicin, artemisinin and [artemisinin+epirubicin]. EPI: epirubicin; ART: artemisinin.

Table 1. CI values of epirubicin in combination with artemisinin on leukemia cells

1A				1B			
HL-60				HL-60/Dox			
EPI ($\mu\text{mol/L}$)	ART ($\mu\text{mol/L}$)	Fa	CI \pm SD	EPI ($\mu\text{mol/L}$)	ART ($\mu\text{mol/L}$)	Fa	CI \pm SD
1.25	400	0.97	0.91 \pm 0.201	1.25	200	0.48	0.50 \pm 0.239
1.25	800	0.96	1.58 \pm 0.790	1.25	400	0.97	0.18 \pm 0.141
2.5	200	0.98	0.87 \pm 0.614	2.5	100	0.16	0.57 \pm 0.417
2.5	800	0.98	1.61 \pm 0.467	2.5	400	0.91	0.31 \pm 0.710
5.0	200	0.99	1.19 \pm 0.326	5.0	100	0.19	0.54 \pm 0.632
5.0	400	0.99	1.39 \pm 0.842	5.0	200	0.47	0.52 \pm 0.155
Fixed ratio concentrations (EPI:ART=1:160)				Fixed ratio concentrations (EPI:ART=1:80)			
Total dose*		Fa	CI \pm SD	Total dose*		Fa	CI \pm SD
201.25		0.98	0.47 \pm 0.141	101.25		0.14	0.59 \pm 0.945
402.5		0.98	1.05 \pm 0.305	202.5		0.46	0.52 \pm 0.557
805		0.98	2.10 \pm 0.610	405		0.92	0.29 \pm 0.835

*Total dose of [epirubicin+artemisinin] in fixed ratio concentrations.

EPI: epirubicin; ART: artemisinin; Fa: median effect; CI: combination index; CI<0.9 denotes synergism, CI=0.9-1.10 – additive effect, and CI>1 – antagonism.

Table 2. DRI values of ART and EPI on HL-60 cells at a fixed ratio (160:1)

Fa	ART ($\mu\text{mol/L}$)	EPI ($\mu\text{mol/L}$)	DRI _{ART}	DRI _{EPI}
0.25	222.9	0.708	2.93	5.8
0.35	252.4	0.83	2.08	4.3
0.50	296.3	1.03	2.60	5.7
0.60	329.1	1.19	1.36	3.07
0.70	369.0	1.39	8.24	1.93
0.75	393.9	1.52	0.002	5.51
0.90	523.6	2.21	0.203	5.36

DRI: Dose reduction index; DRI>1 denotes favourable reduction of the dose; EPI: epirubicin; ART: artemisinin.

the dose of ART is also possible in the combinations that reach up to 70% inhibition. This means that we do not need to use concentrations of ART more than 400 $\mu\text{mol/L}$ when EPI is applied in smaller concentrations (1.52 $\mu\text{mol/L}$). The results from the computer-simulated “dose-effect” correspond adequately to the synergistic effect of ART and EPI when applied in smaller concentrations – 200 and 1.25 $\mu\text{mol/L}$, respectively.

Comparative analysis on the basis of IC₅₀

EPI and ART reach 50% inhibition on the HL-60 cells in near concentrations in both the separate and combined treatment (Comfix) (Table 3). However, while EPI 5

Table 3. Bioequivalent IC₅₀ concentrations of artemisinin, epirubicin and the fixed-ratio combinations on HL-60 and HL-60/Dox

IC ₅₀ ($\mu\text{mol/L}$)	HL-60 \pm SD	HL-60/Dox \pm SD
Artemisinin	307.7 \pm 33	360.2 \pm 40
Epirubicin	1.25 \pm 0.5	>>5
Comfix 1:160*	[1.03 \pm 0.5 + 296.3 \pm 10.5]	---
Comfix 1:80*	---	[2.4 \pm 0.25+190.1 \pm 40]

*[epirubicin+artemisinin] according to COMPUSYN analysis.

$\mu\text{mol/L}$ does not influence the MDR HL-60/Dox cells alone, it is effective at 2.4 $\mu\text{mol/L}$ when used in combination with 190 $\mu\text{mol/L}$ ART. Moreover, this dose for ART is 1.8 times smaller than the one when ART is applied alone. Thus, the evaluated ratio of EPI:ART equal to 1:80 proves to be a better choice for applied treatment of the HL-60 cells. Interestingly, the combined treatment with ART and EPI on HL-60/Dox resistant cells causes 50% inhibition in smaller concentrations than these on HL-60 cells.

Modulation effect of artemisinin on iron-induced oxidative molecular damage

Lipid peroxidation assay

Some extent of oxidative molecular damage was evident in all samples, including the one only with the oxidizable substrate lecithin (1 mg/ml) where only autoxidation processes triggered by the experimental conditions were expected

(Fig. 2). The measured absorbance of this sample was 0.092 which is corresponding to 22.15% molecular damage when compared to the control. The extinctions of the samples containing ART and lecithin were a little bit higher compared to the one containing only lecithin but no statistically significant differences were observed. In the presence of iron and ART the absorbance values of the samples were more than three times higher compared to the sample containing only ART. The extent of molecular damage observed in the three tested concentrations of ART (96.05% to 102.74%) was identical to the control.

2-deoxyribose degradation assay

The extent of the observed molecular damage in the different samples depended on a number of factors – sample constituents and the used concentration of ART (Fig. 3). In the sample containing only 2-deoxyribose, minimal increase of the absorbance value at 532 nm was observed. This indicates negligibly low extent of 2-deoxyribose degradation triggered by the applied experimental conditions. In the sample containing the used substrate and ART (0.95 mmol/l) the observed absorbance value was more than 20 times higher compared to the samples containing

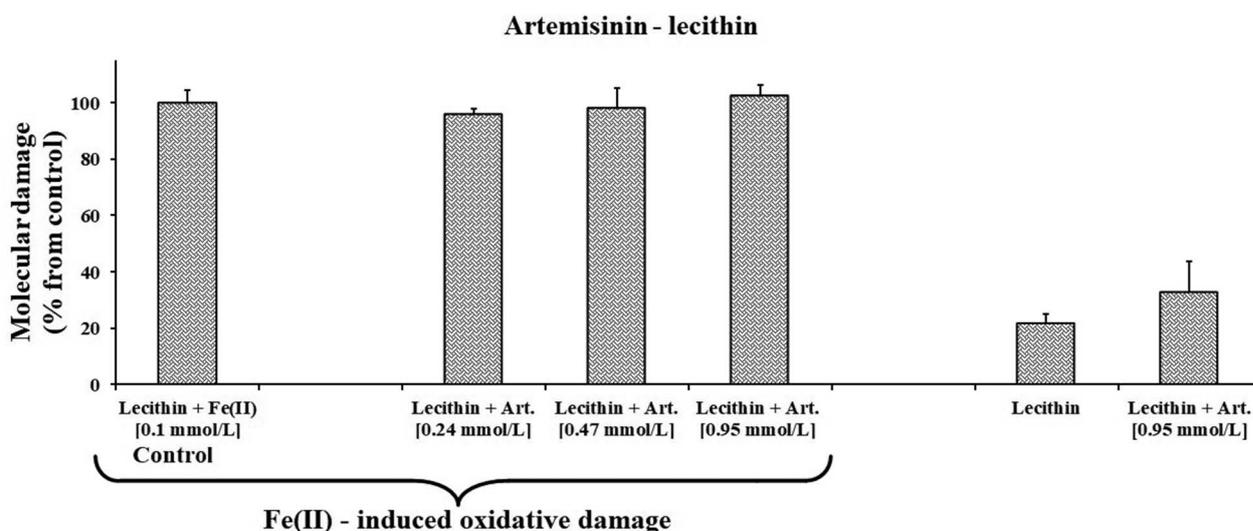


Figure 2. Degree of lecithin oxidative molecular damage. Lecithin (1 mg/ml) was incubated with 0.1 mmol/L Fe(II) in the absence or presence of artemisinin. Data are reported as means ± SD of percentage of control for 3 separate measurements.

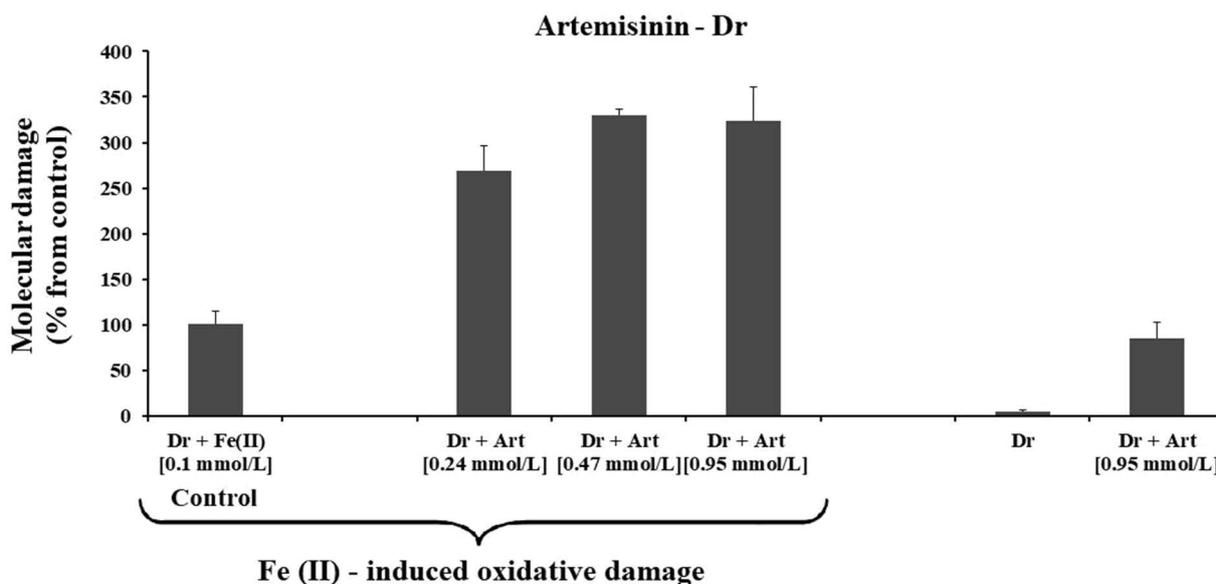


Figure 3. Degree of 2-deoxyribose oxidative molecular damage. Deoxyribose (0.5 mmol/L) was incubated with 0.1 mmol/L Fe(II) in the absence or presence of artemisinin. Data are reported as means ± SD of percentage of control for 3 separate measurements. Dr: 2-deoxyribose; Art: artemisinin.

only the 2-deoxyribose. The estimated molecular damage was 84.65%, close to the observed in the control samples. In the presence of both iron and ART the absorbance values of the samples were two to three times higher compared to the control. These results suggest an increase of TBARS products, deoxyribose degradation and molecular damage.

DISCUSSION

Combination chemotherapy is the main approach in treating resistant and advanced malignant tumors. In this study we comparatively evaluated the tumor-inhibiting effect of artemisinin, epirubicin and the combination of the two drugs at varying and fixed-ratio concentrations on sensitive and MDR-resistant HL-60 cell lines. Artemisinin potentiates epirubicin-induced cytotoxicity on HL-60/Dox cells as revealed by the reached tumor inhibition and the CI values (**Fig. 1B**; **Table 2**). Comparison between the dose-response on the sensitive HL-60 and the resistant leukemia cells shows increase of the tumor inhibition on HL-60/Dox cells in dependence of the applied concentrations of ART. Our results confirm that ART has a modulating effect against EPI on resistant tumor cells.

Some investigators obtained antagonism between artemisinin and doxorubicin on HT29 colon cancer cells and MCF-7 breast cancer cells due to the P-gp expression when artemisinin was applied as pre-treatment.¹⁶ But it is known that the effect of the combination treatment depends not only on the kind of the components but also on the type of tumor cells, tested concentrations and the mechanism of its antitumor action, including presence of Fe²⁺ needed for bioactivation of the bioreductive drugs. However, detailed investigations of different tumor cell models are required for a definitive conclusion. In this aspect, the application of the quantitative assay based on the mathematical algorithm of Chou Talalay^{19,20} may improve the experimental design of the combination therapy. In our study we define that the combination of EPI with ART is highly effective and makes it possible to apply lower EPI concentrations than IC₅₀. In addition, we obtained that the ratio between EPI and ART of 1:80 is synergistic (CI<0.59) and effective on resistant HL-60/Dox cells, reaching 92% anti-proliferative effect. This may be important as a prognostic factor for the treatment of resistant human tumors.

The study of artemisinin in the lecithin-containing system demonstrates that the drug does not change the extent of iron-induced oxidative damage of lecithin at in vitro conditions in the presence and absence of iron. This suggests that in this case it is not likely to observe generation of end lipid peroxidation products which are typical for many cytostatic drugs.²² The obtained results are in accordance with other authors' reports who observe that when administered alone ART has no effect on plasma TBARS and the membrane fluidity in in vitro culture and malaria patients.²³

The effect of ART on the 2-deoxyribose degradation was

similar to the one induced by iron. Artemisinin denoted its capability to induce deoxyribose oxidative degradation and exert synergistic effect with iron. The modulation effect of ART (at cell level and oxidative damages) is consistent with the data reported by Wu et al.²⁴ for dihydroartemisinin which sensitized cells to apoptosis induced by doxorubicin. Our results from the lipid peroxidation assay and the deoxyribose degradation assay add to the gathered knowledge for the radical-induced mechanisms of cytotoxicity of artemisinin.

CONCLUSIONS

Artemisinin synergistically increases the epirubicin-induced inhibition of HL-60/Dox resistant cells when applied in varying or fixed-ratio (1:80) concentrations. The synergism is partially associated with the sensitization of the tumor cells to the cytostatic. Artemisinin shows a favourable dose modulation effect towards epirubicin on sensitive HL-60 cells. In regards to its mechanism of action, our results show that artemisinin induces deoxyribose molecular damage to an extent similar to the iron-induced oxidation, and this effect is even higher when artemisinin is applied in the presence of Fe²⁺. Thus, the combination of [artemisinin+epirubicin] on multi-resistant tumor cells may be a promising strategy for cancer treatment. Nevertheless, further research is needed to define the spectrum of artemisinin's combined antitumor activity.

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Противоопухолевая активность комбинации артемизинина и эпирубицина в лейкозных клетках человека

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Дата получения: 29 июня 2020 ♦ **Дата приемки:** 30 июля 2020 ♦ **Дата публикации:** 31 августа 2021

Образец цитирования: Zhelyazkova MY, Hristova-Avakumova NG, Momekov GTsv. Antitumor activity of the combination of artemisinin and epirubicin in human leukemia cells. Folia Med (Plovdiv) 2021;63(4):488-95. doi: 10.3897/folmed.63.e55938.

Резюме

Введение: Мы оценили подавляющий опухоль эффект артемизинина, вводимого отдельно и в комбинации с эпирубицином, на лейкоэмические клеточные линии HL-60 и HL-60/Dox, его эффект модуляции дозы и его способность влиять на вызванное железом окислительное повреждение биологически релевантных молекул.

Материалы и методы: Для демонстрации ингибирования пролиферации опухолевых клеток и оценки синергетического и модулирующего действия артемизинина и эпирубицина в различных концентрациях использовали МТТ-тест и метод Chou-Talalay Кроме того, мы использовали спектрофотометрический анализ для определения способности артемизинина влиять на индуцированную железом молекулярную деградацию лецитина и дезоксирибозы.

Результаты: Артемизинин показал ингибирующее действие на опухоль как на антрациклин-чувствительные, так и на антрациклин-устойчивые клеточные линии промиелоцитов, достигая 88% и 61% (T/C), соответственно, при дозозависимом введении в более высоких концентрациях. Комбинация артемизинина и эпирубицина выявила синергетический эффект при всех концентрациях, изученных на доксорубин-резистентных клетках (DI<0.7). Артемизинин сенсibiliзирует эпирубицин-резистентные клетки, что подтверждается значениями доверительного интервала (ДИ), и имеет дозо-модулирующий эффект, как видно из DRI. Артемизинин вызывает окислительную деградацию дезоксирибозы при введении отдельно и обладает синергическим эффектом деградации дезоксирибозы при введении с железом. Однако артемизинин не влиял на исследуемую модельную систему, содержащую лецитин, и не обладал потенциалом индуцировать перекисное окисление липидов.

Заключение: Это исследование представляет новую возможность для повышения эффективности схем лечения на основе эпирубицина с добавлением артемизинина для резистентных опухолей.

Ключевые слова

комбинированная химиотерапия, дезоксирибоза, HL-60 / Dox, лецитин, синергизм