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Original article

THE ANTIMALARIA DRUG ARTEMISININ DISPLAYS STRONG CYTOTOXIC EFFECT ON LEUKAEMIA LYMPHOCYTES IN COMBINATION WITH VITAMIN C AND PRO-VITAMIN K3

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Summary

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This study investigated the anticancer effect of the anti-parasitic drug artemisinin in combination with two redox modulators: vitamin C and pro-vitamin K3 (C/K3) The experiments were conducted on leukaemia cells Jurkat. Cells were treated with either artemisinin or C/K3 alone and with all three compounds. Cell proliferation and viability were analysed using trypan blue stating and automated cell counting. The results showed that artemisinin (>10 μM) suppressed cell proliferation activity, but did not induce cell death up to 500 μM. The drug demonstrated a clear cytostatic effect at concentrations 250– 500 μM – Jurkat cells did not proliferate, but were alive. The combination C/K3 (200:2, 300:3 μM/μM) applied alone did not affect cell proliferation and viability. Vitamins C/K3 in concentration ratio 500:5 (μM/μM) decreased cell proliferation activity by ~10%. The triple combination artemisinin/C/K3 manifested synergistic anti-proliferative effects at all concentration ratios analysed. This synergistic effect increased with increasing C/K3 concentration. Based on literature data, it was assumed that the anti-proliferative effect of the triple combination was mediated by changes in the redox-homeostasis of cancer cells. The C/K3 redox system likely acted on cancer mitochondria and increased superoxide production and activation of pro-apoptotic signals, specific for cancer cells. On the other hand, artemisinin could generate hydroxyl radicals as a result of activation of Fenton reactions, depleting intracellular reducing equivalents. Both redox mechanisms lead to activation of signal pathways for induction of cancer cell death.

Key words: artemisinin, cancer, pro-vitamin K3, redox-homeostasis, vitamin C

INTRODUCTION

The parasitic diseases are a serious health problem in many countries, including Bulgaria, due to the increased prevalence of animal and human infections. The parasitoses most widespread at a global scale: echinococcosis, trichinellosis, visceral leishmaniasis, malaria, are of paramount importance and could be life-threatening for patients with immunodeficiency disorders, as well as for patients undergoing anticancer therapy (Brown & Neva, 1995).

The International Agency for Research on Cancer (IARC) identifies ten infectious agents (including viruses, bacteria, parasites) which are able to induce cancer diseases (Benamrouz *et al.,* 2012). Although malaria *per se* does not appear to be involved in carcinogenesis, it is strongly associated with the occurrence of endemic Burkitt lymphoma in areas holoendemic for malaria. The initiation of *Plasmodium falciparum* related endemic Burkitt lymphoma requires additional transforming events induced by the Epstein-Barr virus (van Tong *et al.,* 2017). It is interesting to note that there are over 200 species of malaria plasmodia. Humans are infected by five but birds, bats, lizards, rodents, antelopes and many other animals are also hosts for malaria parasites (Petney & Andrews, 1998). This relationship between malaria and cancer diseases gave us a reason to study the anticancer activity of an antimalarial drug and to try to find a synergistic effect with other natural compounds.

Artemisinin, isolated from the leaves of the herb *Artemisia annua L.* (sweet wormwood), is a medicinal plant used in Chinese medicine and other countries in Asia and Africa to treat chills and malaria (Klayman, 1985; Price *et al.*, 1996; Wang *et al.*, 2010). In addition to anti-parasitic effect, artemisinin shows anticancer activity (Efferth & Volm, 2005; Yang *et al*., 2014; Li *et al.*, 2018; Zhang *et al.,* 2018). However, the exact mechanism of its anticancer effect is not clear. Studies have demonstrated that artemisinin directly attacks mitochondria and impairs their function (Wang *et al*., 2010; Efferth, 2017; Li *et al.*, 2018) due to its specific chemical structure (a sesquiterpene lactone bearing a peroxide group and lacking nitrogen-containing heterocyclic ring system). This is accompanied by overproduction of reactive oxygen species (ROS), disturbance of intracellular redox homeostasis and activation of apoptotic pathways in cancer cells (Gao *et al*., 2013; Ganguli *et al*., 2014; Greenshields *et al*., 2017; Li *et al*., 2018). Similar mechanisms are described in parasites (Meshnick *et al.*, 2002; Wang *et al.*, 2010).

Conventional cancer chemotherapy and radiation therapy may have limited efficacy due to development of drug resistance and harmful side-effects on normal cells and tissues via disruption of redox-homeostasis (Zhang *et al*., 2018). In the last ten years, numerous studies have demonstrated induction of synergistic cytotoxic effects of combined application of drugs and redox modulators on cancer cells (Wondark, 2009; Romero-Canelón *et al*., 2015; Alshatwi *et al*., 2016; Chen *et al*., 2016). Some of them demonstrate an induction of ROS-independent cell death in cancer cells and lack of cytotoxicity on normal cells and tissues (Pathak *et al*., 2005; Simons *et al*., 2007; Lou *et al*., 2015; Zhelev *et al*., 2015, 2016, 2017). These findings could be used as a basis for development of new therapeutic strategies to minimise harmful side-effects of conventional anticancer therapy (Ivanova *et al*., 2016). It was recently reported that

artemisinin sensitises different cancer cell lines to conventional anticancer drugs, and these effects are potentiated by combining with natural products or their synthetic derivatives (Efferth *et al.*, 2015).

Our study was focused on the potential cytotoxic effect of artemisinin on leukaemia cells, after co-adminsitration with vitamin C and pro-vitamin K3 (C/K3). It is generally acknowledged that the combination of vitamin C and pro-vitamin K3 is a powerful redox system, which could specifically influence redox homeostasis of cancer cells and parasites (Sakai *et al*., 2012; Desoti *et al*., 2015; Ivanova *et al*., 2018). Recently, we reported that C/K3 sensitises cancer cells to conventional and new generation anticancer chemotherapeutics without significant effects on viability of normal cells (Ivanova *et al.*, 2018).

MATERIALS AND METHODS

Animal research ethics

The present study does not report on or involve the use of any animal or human data or tissue.

Cells and treatment protocol

The experiments were performed on cancer cells Jurkat, derived from patients with acute lymphoblastic leukaemia. The cells were cultured in RPMI-1640 medium, supplemented with 10% heat-inactivated foetal bovine serum (FSB) and antibiotics (100 U/mol penicillin and 100 μg/mol streptomycin), in a humidified atmosphere at 37 ºC with 5% CO_2. All cells were collected by centrifugation (1000×g for 10 min) and replaced in a fresh medium without antibiotics, before treatment with the subsequent substance (artemisinin, vitamin C, pro-vitamin K3).

Artemisinin and pro-vitamin K3 were dissolved in dimethyl sulfoxide (DMSO); suitable for cell culture and vitamin C – in phosphate-buffered saline (PBS; 10 mM, pH 7.4). The final concentration of DMSO in the cell suspension did not exceed 1%. At this concentration, DMSO did not influence cell viability.

The drugs, alone or in combinations, were initially applied to the cells (1×10^6 cells/mL) in the concentrations mentioned below (a single dose) and incubated at different time intervals in a cell incubator (24–72 h). Our previous studies on Jurkat cells have indicated that the best cytotoxic effect after combined treatment with drugs and redox modulators were expressed after 48-hour incubation (Zhelev *et al*., 2015, 2016, 2017). At this time interval, aliquots were used for cell viability assay in the present study.

Cell proliferation and viability assay

Cell proliferation and viability were analysed using trypan blue staining and Countess™ Automated Cell Counter (Invitrogen) at very precise standardisation of the measurements. Three independent experiments (with two repetitive measurements) for each experiment were done for each sample. Untreated cells were used as controls.

The cells were incubated with either C/K3 or artemisinin alone or artemisinine/C/K3 at the following concentrations: 50 μM and 100 μM artemisinin; 200 μM, 300 μM and 500 μM vitamin C; and 2 μM, 3 μM and 5 μM pro-vitamin K3.

Statistical analysis

All results are expressed as mean ± standard deviation (SD) from two independent experiments with three parallel samples for each point per experiment (n=3). Comparisons between the groups were

performed using Student's *t*-test. A value of $P<0.05$ was considered significant.

RESULTS

The concentration-dependent effect of artemisinin on the proliferation activity and viability of leukaemia lymphocytes is presented on Fig. 1. The cancer cells were treated with different concentration of artemisinin (from 1 to 500 μM) and the cell proliferation and viability were measured after 48-hour incubation. The results show that artemisinin (>10 μM) suppressed cell proliferation activity, but did not induce cell death up to 500 μM (Fig. 1A). Artemisinin showed a clear cytostatic effect at concentrations 250–500 μM – Jurkat cells did not proliferate, but were alive (Fig. 1B).

The aim of this part of the study was to select the optimum concentrations of artemisinin, inducing ~20-30% decrease of cell proliferation compared to control (untreated) cells, in order to analyse the potential synergistic or antagonistic effects after co-administration with C/K3. It was found out that at concentrations of 50 μM and 100 μM artemisinin decreased proliferation activity of Jurkat cells by ~15% and ~30% respectively (Fig. 1B). These concentrations were used in the

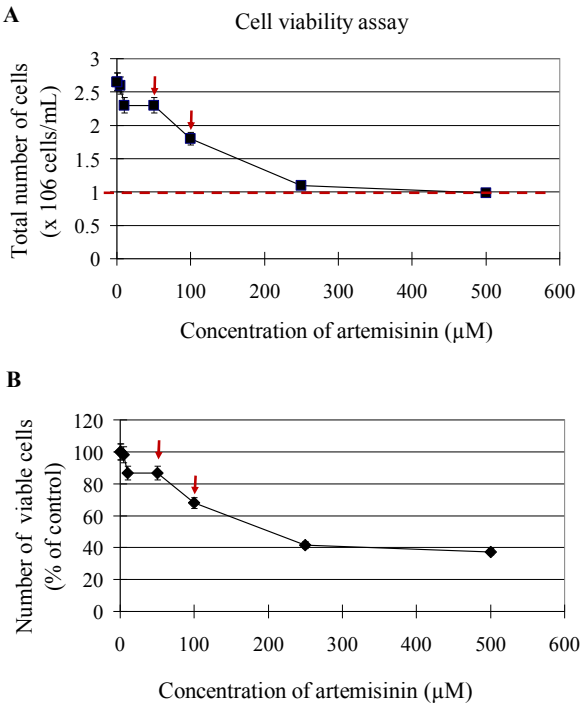

Fig. 1. A. Proliferation activity of leukaemia lymphocytes (Jurkat) after 48-hour treatment with artemisinin in different concentrations. The dotted line indicates the number of untreated cells at the start of the experiment (1×10^6 cells/mL). The arrows indicate artemisinin concentrations selected for further experiments. **B.** Number of viable cells after 48-hour treatment with artemisinin at different concentrations, presented as a percentage of control (untreated) cells. Cell viability in all analysed samples was 94–99%. Data are presented as mean±SD (n=3).

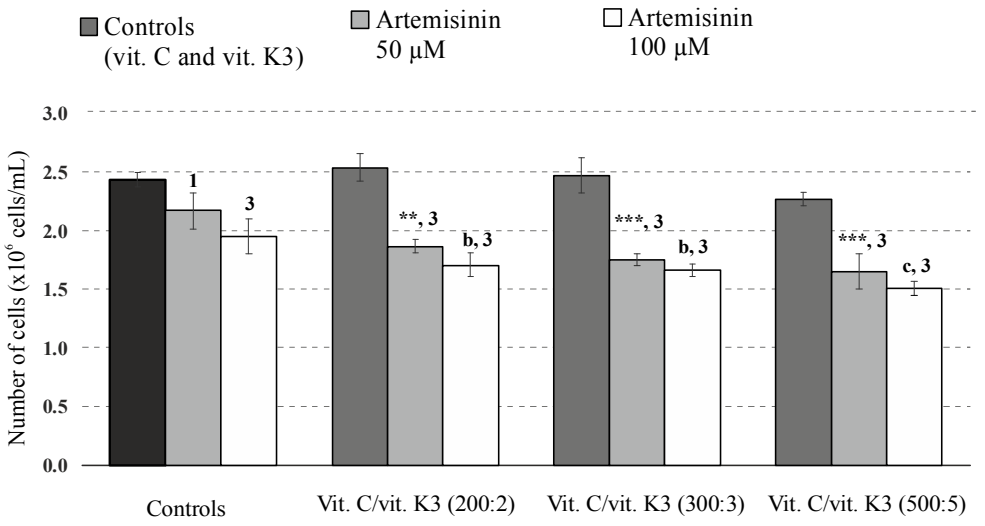

Fig. 2. Effect of vitamins C/K3 applied in different concentration ratios either alone or in combination with 50 μM or 100 μM artemisinin on cell proliferation and viability of leukaemia lymphocytes (Jurkat) after 48-hour incubation. The black bar indicates untreated cells (controls). Cell viability in all analysed samples was 92– 97% (mean±SD, n=3). Levels of statistical significance: ** P<0.01; ***P<0.001 between 50 μM artemisin and 50 μM artemisin + C/K3; ^{b}P<0.01; ^{c}P<0.001 between 100 μM artemisin and 100 μM artemisin + C/K3;^{1}P<0.05; ^{3}P<0.001 between 50 μM and 100 μM artemisin with/without C/K3 and controls.

next experiments to investigate the potential of vitamins C/K3 to sensitise leukaemia cells to artemisinin.

The effects of artemisinin (at 50 μM and 100 μM) plus C/K3 on leukaemia lymphocytes are presented on Fig. 2. The combination of vitamins C/K3 (200:2, 300:3 μM/μM) applied alone did not affect cell proliferation and viability. Vitamins C/K3 at concentrations 500:5 (μM/μM) decreased cell proliferation activity by ~10%. The triple combination artemisinin/C/K3 reduced cancer cell proliferation and displayed anti-proliferative effects at all tested concentration ratios. It was observed that the effects increased parallelly with increasing C/K3 concentration (Fig. 2).

DISCUSSION

In general, the results of the present studies indicated anticancer activity of the anti-malaria drug artemisinin, applied on leukaemia lymphocytes after evaluation of its effect at different concentrations on Jurkat cancer cell line through measurement of cell viability. A dose-dependent suppression of cancer cell proliferation was demonstrated. There are data, which also displayed similar antiproliferative effect of artemisinin and its derivatives on different cancer cell line types. For example, Fox *et al.* (2016) established that artemisinin-related compounds (artemisinins) had a potent antineoplastic activity on 23 tested acute leukaemia cell lines. They observed reduced cell proliferation and clonogenicity, induced apoptosis, and

increased intracellular levels of reactive oxygen species (ROS) in all leukaemia cell lines, treated with artemisinins, applied at submicromolar concentrations. Another study indicated that artemisinin induced apoptosis in non-small cell lung cancer cells (A549 cells) (Gao *et al.*, 2013). The review article of Zhang *et al.* (2018) collected various scientific data, demonstrating that artemisinin-related compounds had cytotoxic effects against a variety of cancer cells through pleiotropic effects, including inhibiting the proliferation of tumour cells, promoting apoptosis, inducing cell cycle arrest, disrupting cancer invasion and metastasis, preventing angiogenesis, mediating the tumour-related signalling pathways, and regulating tumour microenvironment. It was also emphasised on the fact that artemisinins demonstrated minor side effects to normal cells and manifested an ability to overcome multidrug resistance which is commonly observed in cancer patients (Zhang *et al.,* 2018). In this connection, Ding *et al.* (2018) observed that cancer cells contained significantly more intracellular free iron, compared to normal cells. They made a conclusion that elevated free iron levels provided a suitable condition for artemisinin to form cytotoxic free radicals due to the induction of Fenton reactions (Ding *et al.,* 2018). Although antitumour mechanism of artemisinin action is still unclear, recent studies suggest that the endoperoxide bridge (antimalarial chemical group) plays a crucial role in its anticancer activity. It is involved in elevated ROS production, impact on redox homeostasis, specific in cancer cells and subsequent induction of apoptosis (Gao *et al*., 2013; Ganguli *et al.,* 2014), ferroptosis (Eling *et al*., 2015), necrosis (Button *et al.,* 2014), and other dysfunctions in cancer cells (Yang *et al.,* 2014; Greenshields *et al.*, 2017).

For the first time, our results demonstrated increased sensitising of leukaemia lymphocytes induced by artemisin after its combination with vitamin C and pro-vitamin K3. It is also observed that suppression of cancer cell proliferation due to the anti-malarial drug was stronger with increasing C/K3 concentration ratio: it was the most noticeable when C/K3 was applied at concentrations 500:5 (μM/μM). They are few studies reporting synergistic cytotoxic effect of artemisinin, applied on leukaemia lymphocytes. A similar anticancer potential was reported by Elf *et al.* (2017) through investigation of co-application of 6-phosphogluconate dehydrogenase inhibitors and dihydroartemisinin, leading to synergistic cytotoxicity and attenuation of tumour growth in cancer-bearing mice (xenografts of human K562 leukaemia cells) due to up-regulation of AMP-activated protein kinase. However, at the same time, no significant toxicity neither on normal haematopoietic cells of the same mice, or on red blood cells and mononucleocytes from healthy human blood donors, exposed to combined treatment with 6-phosphogluconate dehydrogenase inhibitors plus dihydroartemisinin were observed (Elf *et al.,* 2017).

Based on our previous results, as well as other scientific data, we considered that the specific influence of artemisinin and C/K3 combination on cancer cellular redox homeostasis by could be a possible approach for reduction of cancer proliferation. Like the antitumour mechanism of action of artemisinin, the combination of vitamin C and pro-vitamin K3 is renowned as a powerful redox system with effect on cancer cells and parasites (Sakai *et al.,* 2012; Desoti *et al.,* 2015; Ivanova *et al.,* 2018). Unfortunately, the exact

mechanism of C/K3 action is also under investigation, but various redox mechanisms have been reported (Zhang *et al.,* 2001; Verrax *et al.,* 2004; Bonilla-Porras *et al,.* 2011). It was found that C/K3 serves as electron transfer mediator between coenzyme Q and cytochrome C in the mitochondrial electron transport chain (McCord & Fridovich, 1970; Wagner *et al.,* 1974; Eleff *et al*., 1984; Matsui *et al*., 2015). This is accompanied by an increased generation of superoxide, a shift from anaerobic (glycolytic) to aerobic (oxidative) metabolism and a decrease of hypoxia and lactic acidosis (May *et al.,* 2003; Bonilla-Porras *et al.,* 2011; McGuire *et al.,* 2013a; 2013b; Bonuccelli *et al.,* 2017). Vitamins C/K3 can also affect the ratio between oxidised and reduced forms of the main endogenous redox pairs (NAD^+/NADH; $NADP^+$/NADPH; GSH/GSSH, etc.) and can be directly involved in the function of key enzymes of the glycolytic pathway (lactate dehydrogenase and pyruvate dehydrogenase), glutathione-dependent enzymes etc. (Ivanova *et al*., 2016). Many *in vitro* studies reported the cytotoxic effects of vitamins C/K3 on different cancer cell lines, without affecting cell viability of normal cells (Calderon *et al*., 2002; McGuire *et al.,* 2013). For example, a previous study of ours demonstrated suppression of proliferation of leukaemia lymphocytes by a combination of vitamins C/K3, applied alone at different concentrations, without significant effect on viability of normal lymphocytes up to 500:5 (μM/μM) ratios (Ivanova *et al.* 2018). However, vitamins C/K3 showed impressive synergistic anti-proliferative and cytotoxic effects on leukaemia lymphocytes in combination with conventional anticancer drugs (e.g., barasertib and everolimus) (Ivanova *et al*., 2018). Similar specific anticancer potential of C/K3 was reported by several scientific groups. Zhang *et al*. (2001) demonstrated that cancer cells (HSC-2, HSC-3, HL-60) were more sensitive to C/K3, as compared to normal cells. Vita *et al.* (2011) reported a better cytotoxicity of C/K3 on glioma cells than on normal cells. All presented data, as well as many other studies indicated that the different impact of artemisinin and vitamins C/K3 on viability of cancer and normal cells can be explained, at least partially, by differences in their cellular redox-homeostasis (Ivanova *et al.,* 2016). The redox process differences occurring in cancer and normal cells, are discussed as a key reason for their distinction. In this regard, the vitamins C/K3 likely act specifically on cancer mitochondria to increase superoxide production and activation of pro-apoptotic signals. On the other hand, artemisinin could generate hydroxyl radical as a result of activation of Fenton reactions, which will deplete intracellular glutathione (GSH). Both redox mechanisms could have an impact on activation of intracellular signal pathways, specific for cancer cells and lead to induction of cancer cell death, without affecting cellular viability of normal cells.

In conclusion, the results obtained in our study demonstrated that vitamins C/K3 increased the sensitisation of artemisinin (at 50 μM and 100 μM) and displayed anti-proliferative effects on leukaemia lymphocytes. Based on the data discussed above, the effect of this triple combination was attributed mainly to changes that had occurred in the cellular redox homeostasis during the carcinogenesis. The results of the present study could be successfully applied in human as well as in veterinary medical practice to achieve an optimum effect from treatment of cancer diseases as well as malaria in humans and animals.

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