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Antioxidant and prooxidant properties of artemisinin and epirubicin on *in vitro* biophysical models

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Some natural compounds may exert both antioxidant and pro-oxidant effects, depending on the factors of their environment. This study aims to investigate and compare the effects of the natural substances Artemisinin and Epirubicin using different model systems. A spectrophotometric evaluation of the reduction activity of the tested compounds against stable free radicals ($ABTS^{\bullet+}$ and $DPPH^{\bullet}$) and chemiluminescent evaluation of the scavenging activity against the superoxide anion radical ($O_2^{-\bullet}$) and the hypochlorite (OCl^-) were performed. Both compounds demonstrated good scavenging activity against the stable free radicals. In the system containing OCl^- radical both of the tested compounds decreased the chemiluminescent response and demonstrated a capability to diminish the concentration of the generated in the system reactive oxygen species (ROS). However, the compounds exhibited a different behavior in the $O_2^{-\bullet}$ containing system. In the samples containing artemisinin higher chemiluminescent lightening was observed compared to the controls, whereas in the epirubicin containing samples again a scavenging potency was demonstrated and a decreased chemiluminescent response.

Keywords: Artemisinin, Epirubicin, free radicals, antioxidant, prooxidant

INTRODUCTION

Many natural substances exert pleiotropic biological activities including antitumor, antimicrobial, antivirus, immunomodulatory, anti-inflammatory, and antioxidant effects. In many cases plant substances show both cytotoxic activity and anti-oxidative effect in a concentration-depending manner [1, 2]. A possible explanation of this divergent behavior may be that evolutionally it is due to some cell mechanisms for protection of the organisms' vitality.

Natural products are a rich source of cytotoxic substances which can generate reactive oxygen species and can go through bio-activation to form quinone and other metabolites to exert a damaging effect on the membranes, proteins and DNA [3]. In this point of view, the bioreductive drugs such as mitomycin C, etoposide, bleomycin, the antitumor anthracyclines and artemisinins are amongst the most appropriate examples. All these compounds share some similar features of the conditions needed for their conversion into potent cytotoxins - the necessity of hypoxic environment for their bioactivation and the involvement of some specific reductases in the process [4]. According to reported in the literature cases, one of the initial steps associated with the reduction process results in the generation of a transient intermediate (in most of the cases – one-electron product) which subsequently is being effectively back-oxidized by the molecular oxygen. In some cases, the cytotoxicity is not only towards the tumor cells but also to the normal ones.

Epirubicin is an antitumor anthracycline drug derived from Streptomyces species. Its antineoplastic effect is being attributed to its capability to induce intra- and interstrand DNA crosslinks, to poison the topoisomerase II enzyme and intercalate into the double stranded DNA molecule of the proliferating cells which is subsequently related with inhibition of tumor growth through cell cycle arrest and apoptosis [5]. Studies show that in the presence of NADH or NADPH some flavoproteins induce single electron reduction of their molecule, generating a reduced semiquinone radical [6]. However, they also generate oxygen species which damage the cardiac cells [7]. Reactive oxygen species (ROS) generation is being realized by two main possible mechanisms: a non-enzymatic pathway with the implication of iron and an enzymatic pathway using the mitochondrial respiratory chain. Some authors suggest that the mechanisms of cytotoxicity against tumor cells and cardiac cells are different [8].

Artemisinin is a sesquiterpene lactone, isolated from the plant *Artemisia annua*, used as an antimalarial drug, which also exhibits antitumor effects. The free radical generation has been attributed to the presence of a unique trioxane bridge (—C—O—O—C—) in its molecular

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structure which possess the capability to interact with Fe(II) [9]. Artemisinin's cytotoxicity is well documented and is shown to react on both the thiol and amino moieties via iron dependent and independent reactions [10]. The mechanism includes activation of its endoperoxide bond by reduced heme or ferrous iron, which generates carbon-centered radicals (famous as strong alkylating agents) and/or ROS which according to the described in the literature experiments are of major importance for the compound's cytotoxic and antineoplastic activities [11]. Despite the numerous research findings and the identification of several potential targets for the drugs' action, its actual mechanism remains ambiguous [12].

The aim of our study is to investigate and compare the dose-dependent effects of the natural compounds Artemisinin and Epirubicin on the concentration of stable free radicals and different types of ROS by performing a spectrophotometric evaluation of their scavenging activity against the stable ABTS radical cation and DPPH radical and chemiluminescent evaluation of their capability to diminish the concentration of the hypochlorite and the superoxide anion radical.

EXPERIMENTAL

For the evaluation of the anti-radical properties of the tested cytotoxic molecules spectrophotometric and chemiluminescent model systems have been used. We performed spectrophotometric evaluation of the capability of both substances to scavenge the stable free radicals ABTS (2,2-azinobis-(-3-ethyl-benzothiazoline-6-sulfonic acid) and DPPH (1,1-diphenyl-2-picrylhydrazyl) and chemiluminescent investigation of their potency to decrease the concentration of biologically important ROS less or more associated with the neoplastic processes.

Spectrophotometric methods – a spectrophotometer Shimadzu UV-260 has been used for this part of the experimental work. A fresh radical solution has been prepared for each of both assays. Two groups of samples were prepared – control samples where the tested pharmaceutics were omitted and samples containing the studied cytotoxic substances in different concentrations. On the base of the obtained absorbance values for the controls and the samples containing the studied remedies the AOA and the EC_{50} values (the concentration providing 50% AOA) were calculated.

ABTS method – the experiments were executed following the method as described by Re et al. [13]. The initial step comprises preparation of the ABTS radical cation working solution. For this purpose 14 mM ABTS stock solution was mixed with potassium persulfate 2.45 mM (final concentration). Before use this mixture should be kept in the dark at room temperature for 12-16 h. The next step comprises dilution of the obtained suspension with pH 7.4 phosphate buffer saline (PBS) in order to obtain final solution with initial absorbance 0.70 ± 0.01 units at 734 nm. The decrease in the absorbance of 2 ml of $ABTS^{\bullet+}$ after addition of different concentration of the tested remedies was measured at 734 nm exactly 1 hour after the mixing.

DPPH method – The $DPPH^{\bullet}$ scavenging activity was determined as initially reported by Goupy et al. [14]. An ethanol solution with absorbance of 1 was read at 518 nm of the $DPPH^{\bullet}$ was prepared. The tested pharmaceuticals at different concentrations were allowed to react with 2 ml of it for 60 min at room temperature in the dark. Then the absorbance of the samples was measured at 518 nm.

Chemiluminescent methods – the detection of the chemiluminescent response was done using LKB 1251 luminometer set at 37°C. The experimental equipment comprises AT-type computer connected via serial interface to the luminometer and automatic injector. A MultiUse program ver. 1.08 was used for the collection and the processing of the experimental data. The chemiluminescent response was achieved by determination of the area under the obtained chemiluminescent curve. In each run of samples were included negative controls which are required by the software for the estimation of the chemiluminescent response. The ratio between the chemiluminescent response in the presence and the absence of the investigated substances was termed chemiluminescent scavenging index (CL-SI). It was used as an indicator for the pharmaceutics' scavenging index against the hypochlorite or superoxide anion radical.

Luminol-dependent CL in a system of KO_2 generated superoxide anion radical – the experiments were performed using 1 ml samples 50 mM K_2HPO_4/KH_2PO_4, pH 7.4, buffer containing 0.1 mM luminol, and the tested substances. In the control samples, the remedies were omitted. Due to the fast release of superoxide the CL response was measured immediately after the addition of 20 µl KO_2 solution dissolves in DMSO. It was registered for 1 min every 50 milliseconds after the addition of KO_2.

Luminol-dependent CL in a system of NaOCl produced hypochlorite – One milliliter sample of PBS buffer, pH 7.4, containing 0.1 mM luminol, 0.06 mM NaOCl and the tested substances (or buffer for the control sample) were prepared. The

chemiluminescent lightening was registered for 1 min every 50 milliseconds after the addition of NaOCl.

All the experiments have been performed in triplicate. The obtained results are expressed as a mean ± SD (n = 3) for each of the tested concentration in every performed experiment.

RESULTS AND DISCUSSION

The first step from our investigation comprises the evaluation of the anti-radical properties of epirubicin and artemisinin in model systems containing stable free radicals. Both methods are based on the decolorization of the preformed stable free radical solutions proportional of the extent of the anti-radical properties of the studied substances. The methods are simple, reproducible, sensitive and most importantly applicable for both aqueous and nonpolar organic solvents which gives the possibility to determine the antioxidant capacity of both hydrophilic and lipophilic constituents. Despite these systems' biological irrelevancy due to the fact that these two radicals are not observed in the living cells and no conclusions can be made about the sample potency to eliminate biologically important ROS on the base of the obtained by these assays data, both methods are considered as representative for preliminary screening of broad spectrum of complex samples. Additional advantage is that both radicals have different mechanism of neutralization which is giving some indications concerning the possible mechanism of free-radical scavenging.

The anthracycline drug used for chemotherapy epirubicin has demonstrated a different extent of its capability to decrease the stable free radicals concentration in both tested model systems. Its scavenging activity against $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ raises with the increase of its concentration and a linear dependence concentration-AOA in the micromolar concentration range has been observed. The experiments concerning the anti-radical effect of epirubicin against ABTS were performed in a low concentration range from 0 to 6.8 µmol/L where a significant decrease in the concentration of the radical resulting from the demonstrated good scavenging effect of the tested compounds was noticed. At the maximal tested concentration the AOA was 63%. The determined concentration by the dose-response curves providing 50% AOA – EC_{50} using the ABTS assay was 5 µmol/L. When we compare this result with the one of the antioxidant reference Trolox [5.82 µmol/L] obtained using the same experimental conditions we can conclude that epirubicin exhibits better scavenging activity against ABTS compared to the reference in regard of EC_{50}. Against the DPPH radical epirubicin demonstrated lower reactivity. At the maximal tested concentration of 12.5 µmol/L the corresponding AOA was around 20%. EC_{50} values are not presented because of the relatively weak anti-radical activity in the studied concentration range in order to avoid estimation error.

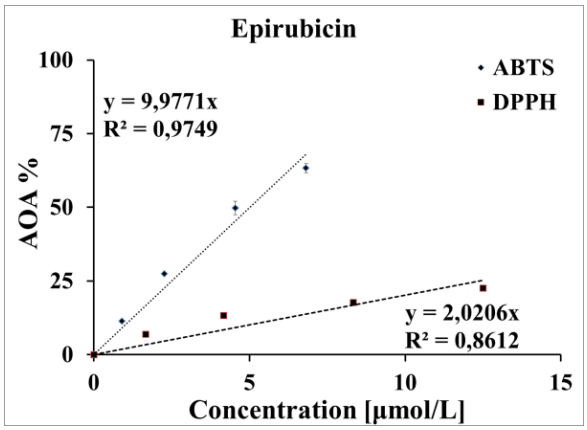

Fig. 1. Concentration dependence of the anti-radical activity of Epirubicin measured using the ABTS and the DPPH assay.

Artemisinin demonstrated scavenging capability against both stable free radicals in the millimolar concentration range. The maximal tested concentration in the ABTS containing systems was 0.98 mmol/L where almost full sample decolorization has been observed and the estimated AOA was over 90%. Using the DPPH assay the observed AOA was close to the one of epirubicin – around 25% but this activity was corresponding to a much higher concentration of 1.7 mmol/L. Again only EC_{50} values for the ABTS system have been estimated – 0.47 mmol/L.

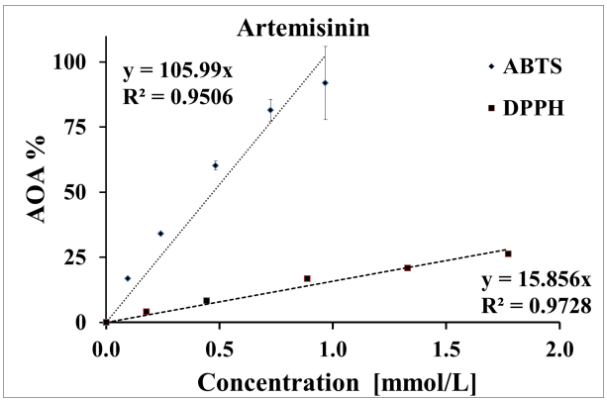

Fig. 2. Concentration dependence of the anti-radical activity of Artemisinin determined in the ABTS and DPPH containing systems.

The obtained data clearly indicates that the tested pharmaceutics possess the capability to diminish the concentration of the stable free radicals in the studied systems. The extent of the observed anti-radical effect depended on the used model systems and the studied substances. Both

investigated drugs denoted better effectiveness against the ABTS radical compared to DPPH. In both assays epirubicin demonstrated better antiradical effect compared to artemisinin.

The evaluation of the potential antioxidant and anti-radical properties may seem at some point surprising having in mind that both drugs are either famous (epirubicin) or investigated as potential (artemisinin) neoplastic agents. This is due to the fact that chemotherapeutic treatment and the use of chemotherapeutic agents induce oxidative stress in the living biological systems which subsequently initiate ultimately necrosis and apoptosis. The antineoplastic drugs induce elevation of lipid peroxidation products which subsequently generate electrophilic aldehydes that can attack many cellular biologically important targets. By far the described in the literature experiments up to this moment have proven that both tested substances are associated with the free radical generation in the human body. Evidences for correlation between this ROS generation and the observed antineoplastic effect (artemisinin) or the observed side effect (epirubicin) have been found.

We chose the stable free radicals systems for our research due to fact that the evaluation of the different pharmacological properties of a lot of remedies and their new designed derivatives (with proven drug-induced oxidative stress and toxicity), comprises as a pilot investigation of their radical scavenging activity evaluation of their potency in model systems containing the stable ABTS and DPPH radicals. The obtained information is useful in the process of analyzing the possible mechanism of free-radical scavenging when combined with other methods like quantum chemical calculations, and in the choice of other test systems for evaluation of potential anti-radical activity. Despite the fact that these results might not be related to the drugs' main indications these results could reveal some additional activities of the substances. They may not cure the pathological condition but will help to ameliorate patients' status or symptoms, widening the knowledge about their properties associated with the patients' tolerability to the drug, adverse effects and even sometimes possibilities for application in other therapeutic areas.

We revealed in the ABTS and DPPH model systems capability of the tested cytostatic molecules to decrease the concentration of the stable radicals with mechanisms of elimination that indicate the substances' capability to exhibit anti-radical properties by SET and HAT motivated us to evaluate their scavenging effect against different type of biologically important ROS (less or more associated with the pathological conditions for which these drugs are being used to). For this purpose we have chosen chemiluminescent methods. The luminol-dependent chemiluminescence has proved itself as an extremely sensitive method. Its main advantages are the low limits of detection and the wide linear dynamic range. In our work, we have chosen to study the scavenging capacity of the tested drugs against the hypochlorite and the superoxide anion radical in order to evaluate the diverse aspects of their activity.

The superoxide anion radicals are known with their lower reactivity compared to other ROS, and a lack of capability to react and initiate oxidative damage to most biological molecules in aqueous solution. The estimation of the anti-radical properties against the superoxide anion is important because it is being considered as a "primary" ROS. The superoxide radical is a substrate for the initial step of the reactions generating other more aggressive ROS. The evaluation of the hypochlorite scavenging activity is related to the estimation of the capability of the tested substances to influence inflammation processes. The concentration of the hypochlorite in the samples corresponds to the one observed during macrophages oxidative burst.

The chemiluminescent evaluation of the scavenging activity of epirubicin denoted capability to decrease the concentration of both – the hypochlorite and superoxide anion radical in the tested systems. The experiments were performed in the micromolar concentration range – in the superoxide containing system from 0 to 100 μmol/L and due to stronger reactivity the tested concentration has been decreased ten times in the hypochlorite one, with the effect being evaluated from 0 to 10 μmol/L. With the increase of the concentration of epirubicin we observed decrease in the chemiluminescent lightening in both systems. At the maximal tested concentration (100 μmol/L) in the superoxide containing system the observed decrease on the CL-SI is 38% compared to the control samples. The estimated from the concentration - CL-SI dependence concentration EC_{50} leading to 50% reduction of the chemiluminescent response was 153.3 μmol/L - beyond the studied concentration range. In the hypochlorite containing system at the highest tested concentration (10 μmol/L) there was a four time decrease of the chemiluminescent lightening compared to the control samples. The CL-SI index was 26.54% and the EC_{50} equaled to 3.77 μmol/L.

Like in the spectrophotometric systems using chemiluminescent methods we again observed decreased capability of artemisinin to diminish the concentration of the presented in the systems

radicals due to which again the experiments were performed in the millimolar concentration range. Different effect has been observed in the presence of the tested compound in both systems. In the superoxide containing system the measured chemiluminescent lightening was higher for the samples containing artemisinin compared to the control ones where the drug was omitted. For the samples containing artemisinin at concentration of 0.32 mmol/L the increase of the CL-SI index compared to the controls was with 12% and at the maximal tested concentration of 1.1 mmol/L the observed elevation was more than 60%.

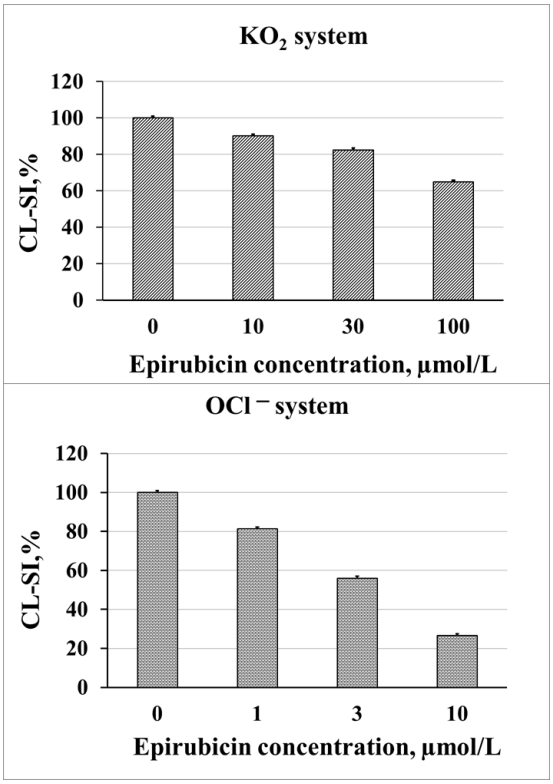

Fig. 3. Epirubicin induced reduction of luminol-dependent chemiluminescence response in model systems with different ROS: KO_2 system – KO_2 (1 mM solution) produced superoxide formation; OCl^- system – NaOCl [0.06 mmol/L] – generated hypochlorite. The assays were carried out using 1 ml samples of 50 mM PBS, pH 7.4, containing 0.1 mM luminol. Results are presented as percentage from the controls as the mean ± SD of one experiment performed in triplicate. Only concentrations where the effect is statistically distinguishable from the control are presented.

No chemiluminescent lightening has been observed in the blank. In the hypochlorite containing system again was observed reduction of the chemiluminescent lightening corresponding to capability to decrease the hypochlorite concentration. Even at the lowest tested concentration (0.01 mmo/L) the observed decrease of the index was more than 20%. At 0.32 mmol/L the CL-SI value was 3.61% indicating full inhibition of the chemiluminescent lightening. The estimated EC_{50} value was 0.034 mmol/L.

The obtained by the chemiluminescent systems results denote that from both tested cytotoxic molecules only epirubicin has the capability to impact the ROS generation process by influencing their initial step i.e. decreasing the concentration of the superoxide anion radical and tackling this way the subsequent reaction associated with the generation of more aggressive and harmful for the cell ROS.

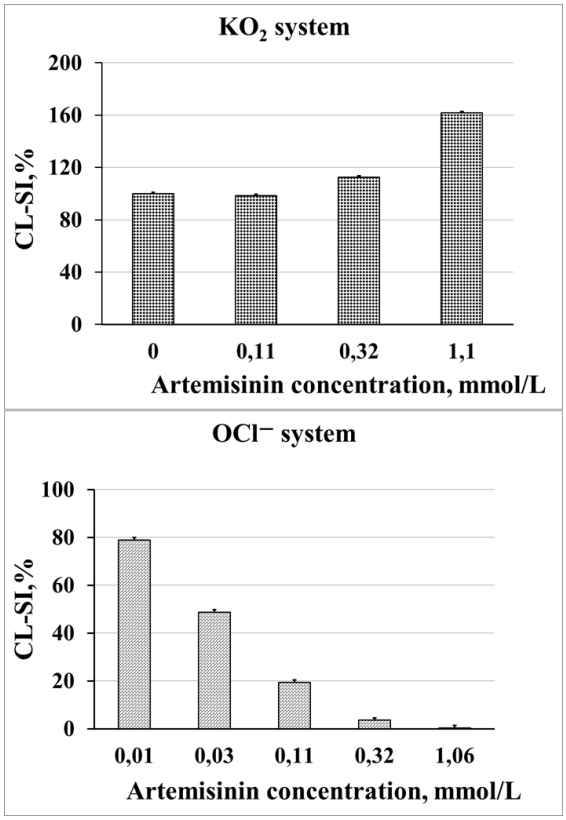

Fig. 4. Artemisinin effect on luminol-dependent chemiluminescence response in model systems with different ROS: KO_2 system – KO_2 (1 mM solution) produced superoxide formation; OCl— system – NaOCl [0.06 mmol/L] – generated hypochlorite. The assays were carried out using 1 ml samples of 50 mM PBS, pH 7.4, containing 0.1 mM luminol. Results are presented as percentage from the controls as the mean ± SD of one experiment performed in triplicate. Only concentrations where the effect is statistically distinguishable from the control are presented.

The observed increase in the CL lightning in the presence of artemisisnin could be attributed to direct interaction of the cytotoxic molecule with the produced in the system superoxide and excited state product formation.

The last should be capable of subsequent interaction with the luminol molecules initiating increase of the lightening in the system. The obtained data suggest possible increase of the *in*

vivo oxidative damage in the presence of artemisinin and superoxide. This effect is of biological importance due to the numerous sources of $O_2^{\bullet-}$ in the living systems (electron transport chain in the inner mitochondrial membrane, xenobiotic-mediated generation or phagocyte NADPH oxidized production) and could be related to its bio-reductive mechanism of cytotoxic activity of artemisinin. Further evaluation of their implication in the observed anti-malaria and cytotoxic activity of artemisninin should be performed. Both substances have demonstrated capability to decrease the hypochlorite concentration which indicates potential capability to influence inflammatory processes.

CONCLUSIONS

The obtained results proved that both tested drugs decrease the concentration of the stable free radicals ABTS and DPPH in chemical *in vitro* model systems – suggesting their capability to exert anti-radical properties by both SET and HAT mechanism. In the containing OCl^-system both – epirubicin and artemisinin decreased the chemiluminescent response in a concentration-dependent way. The compounds exhibited different behavior in the $O_2^{\bullet-}$ containing system. In the samples containing artemisinin was observed higher chemiluminescent lightening compared to the controls, whereas epirubicin again demonstrated scavenging potency by decreasing the chemiluminescent response. In all tested systems epirubicin demonstrated better radical scavenging activity and necessity for lower amount of the tested substance to decrease the radical concentration compared to artemisinin. The observed anti-radical properties confirm the pleiotropic effects of such compounds.

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