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Cytotoxic Effects of Alpha-Lipoic Acid and its Biodegradation Derivatives – a Comparative Study in an *In Vitro* Model of Murine Embryo Fibroblasts

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Various biological activities of alpha-lipoic acid (LA) have recently been reported. In our laboratory two four-carbon products of LA biodegradation had been synthesized: tetranor-dihydrolipoic acid (tetranor-DHLA) and 2,4-bismethylthiobutanoic acid (BMTBA). In subsequent experiments their biological properties were studied in order to assess if LA metabolites might exhibit biological activity in a living organism and show lower/higher toxicity than the parental compound. In particular, the present study investigated antiproliferative activity of LA and its biodegradation derivatives, DHLA and BMTBA, using an *in vitro* cell model of murine fibroblast cell line 3T3-L1. Human fibroblasts and hepatoma cells were employed to compare the effects. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) viability assay was used to measure adequate metabolic activity of cells incubated for 24 h with the addition of the tested compounds, in order to assess the structure-activity relationship. The strongest cytotoxic effect in the 3T3-L1 cell culture was observed after incubation of cells with tetranor-DHLA, the weakest effect was found for BMTBA. As regards LA and its derivatives tested in human fibroblasts, the most toxic compound was BMTBA, while LA was least toxic. The most toxic compound to hepatoma cells Hep G2 was tetranor-DHLA.

Key words: alpha-lipoic acid, cytotoxicity, murine embryo fibroblasts

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INTRODUCTION

Alpha-lipoic acid (LA) is a natural dithiol compound which exhibits very strong antioxidant activity (Bilska and Włodek, 2002; 2005; Dudek et al., 2008). Numerous recent studies have confirmed the beneficial properties of this compound in mammals, e.g. the regulation of the inflammatory response of an organism (Dudek et al., 2013; Zygmunt et al., 2013). Several pharmacological applications of LA were proposed, especially in the treatment of diabetic neuropathy (Gorąca et al., 2011), neurodegenerative diseases (Gorąca et al., 2011), cardiovascular diseases (Dudek et al., 2014), intoxications (Iciek et al., 2011; Sokołowska et al., 2013; 2014) and many others. Also the reduced form of LA, dihydrolipoic acid (DHLA), shows very strong reducing properties (Moini et al., 2002), however DHLA was reported to be more toxic than LA (RTECS), therefore currently LA is being tested for use in medical therapies.

The estimation of drug toxicity is an important step during the discovery process. The main problems, which one should take into consideration, are unpredictable effects of chemicals on mammalian cells and, on the other hand, species and tissue specificity of drug action. The strategy of ADME/Tox studies also includes the estimation of the toxicity of drug biodegradation products. Therefore the present study was performed to elucidate the differences in the cytotoxicity of LA, 2,4-bismethylthiobutanoic acid (BMTBA), which is one of the LA biotransformation products (Schupke et al., 2001; Teichert et al., 2003; Kwiecień et al., 2013) and tetranor-dihydrolipoic acid (tetranor-DHLA), a DHLA analogue. The process of synthesis of the compounds was described by Kwiecień et al. (2013). Biological activities of the newly synthesized compounds, such as their anti-inflammatory properties, have recently been intensively studied (Kwiecień et al., 2013). Figure 1 shows the structures of the tested compounds.

As regards drug design, the testing process usually involves the characterization of compounds in cell-based *in vitro* assays. This step enables screening for the prospective drug features, such as low cytotoxicity and/or specific activity towards tumor cells. The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay is a widely used, standard method for measuring inhibition of cell proliferation in the process of optimizing new anticancer drugs. Since cytotoxicity and effect on the proliferation of cancer cells shown by the tested drugs are one of the most important parameters in cancer drug discovery, we also aimed to find out if the tested compounds exhibited antitumor potency.

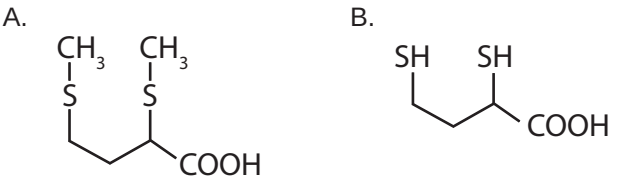

Fig. 1. Chemical structures of LA biodegradation products.
A. – 2,4-bismethylthiobutanoic acid (BMTBA),
B. – tetranordihydrolipoic acid (tetranor-DHLA)

MATERIALS AND METHODS

Chemicals

Resistivity, MΩ•cm at 25°C, deionized water (esistivity, MΩ•cm at 25°C) was obtained from Milli-Ro and Q water purification system, (Merck-Millipore, USA), ethanol was from Merck. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was purchased from Sigma (Germany). Trypan Blue reagent was from Invitrogen (USA). Media and sera were from ATCC (USA), antibiotic mixture and trypsin – 0.05% EDTA solution were from Gibco (USA). Sterile and non-toxic plates, flasks, tips, centrifuge tubes were from Sarstedt (Germany).

Cell culture conditions and MTT assay

Murine embryo fibroblasts (ATCC designation: 3T3-L1, CL-173) were kept in Dulbecco's Modified Eagle's Medium (DMEM). Human hepatocellular carcinoma cells (ATCC designation: Hep G2, HB-8065) and human skin fibroblasts (ATCC designation: BJ, CCL-2522) were cultured in Eagle's Minimum Essential Medium (EMEM).

The media were supplemented with 10% fetal bovine serum and with 1% antibiotic solution (100 IU/ml penicillin, 0.1 mg/ml streptomycin). The cells were subcultivated twice a week using trypsin – 0.05% EDTA solution. Cell viability during the culture was assessed with Trypan Blue Exclusion Test (Trypan Blue solution in buffered PBS without Ca++ Mg++, pH 7.4) in an automatic cell counter (Countess, Invitrogen). The cells in the exponential growth phase were seeded into 96-well plate at a density of 5×10^4 cells/well in 200 µl of medium. For MTT test incubations, the positive control (100% of growth) were the cells cultured in medium with an adequate amount of solvent, ethanol, the negative control was prepared with 20 mM/l of hydrogen peroxide. Six concentrations of lipoic acid dissolved in ethanol as well as LA derivatives (from 10^{-3} M to 10^{-6} M) were tested, each in triplicate. After 24 hours of incubation with the compounds, the media were replaced with fresh media containing additionally MTT (5 mg/ml in PBS, pH 7.4). Then MTT formazan generated during incubation was dissolved and the absorbance was measured at 550 nm (reference wavelenght was 690 nm) using BMG Labtech´s Fluostar Optima microplate reader [Labtech GMBH, Germany] (Pasko et al., 2013; Nunes et al., 2014, Tyszka-Czochara et al., 2014).

Data analysis

The MTT data were tested by one-way ANOVA, followed by Duncan's *post hoc* test. Statistical calculations were carried out using the commercially available packages Statistica v.5.1 (StatSoft, Ic., Tulsa, USA). EC_{80} values were calculated using Omega Data Analysis (Labtech GMBH, Germany).

RESULTS

To assess the structure-activity relationship of LA and LA derivatives, the cytotoxic activities of LA, tetranor-DHLA and BMTBA were determined using the colorimetric MTT assay. In murine (3T3-L1) and human (FB) fibroblasts, the inhibitory potency of all the tested compounds was less pronounced than in human hepatoma cells (Tab. 1).

Table 1. Effects of LA and LA derivatives on murine (3T3-L1) human (FB) fibroblasts and on hepatoma (Hep G2) cells.

	3T3 L1 cell line	FB cell line	Hep G2 cell line
LA	225	281	145
tetranor-DHLA	212	245	142
BMTBA	300	235	150

Cytotoxicity was measured by MTT test and expressed as EC80 (concentrations of compounds [µM] at which 80% cells were viable after 24 h of incubation with the tested compound). One of three independent measurements was presented.

The strongest cytotoxic effect in the 3T3-L1 cell culture was observed after incubation of the cells with tetranor-DHLA (the lowest EC_{80} value), the weakest effect was found for BMTBA. As regards LA and its derivatives tested in human fibroblasts, the most toxic compound was BMTBA, while LA was least toxic. The most toxic compound to hepatoma cells Hep G2 was tetranor-DHLA, but the differences between the tested compounds were not significant.

DISCUSSION

Since the differences in cytotoxic potency between LA and its derivatives had already been reported, it was of interest to asses if different LA metabolites exerted a similar or different effect on mammalian cells. For this purpose, the *in vitro* model of murine (3T3-L1) and human (FB) fibroblasts was established. The effect obtained in physiological cell lines was compared with the effect in tumor cells (Hep G2). The MTT viability assay showed adequate metabolic activity of cells incubated for 24 h with the addition of the tested compounds.

In the case of intraperitoneal infusion of LA in mice, the toxicity was established at 160 mg/kg b.w. and at 100 mg/kg b.w. after the exposure of animals to DHLA (RTECS, 2014). DHLA is a more potent cytotoxic agent than LA acid because of a different cytotoxic mechanism involved (Ya-

MASAKI et al., 2009). In the present experiments using the *in vitro* model of mouse fibroblasts the same tendency was observed, since EC_{80} values were higher for LA as compared with tetranor-DHLA (Tab. 1). As regards human physiological fibroblasts, the trend was also preserved, which may suggest that higher cytotoxicity of DHLA, compared with LA, is maintained through species. Acute toxicity of BMTBA after one intraperitoneal infusion in mice was 588.57 mg/kg b.w., and 75.4 mg/kg b.w. for tetranor-DHLA (KWIECIEŃ et al., 2013). Accordingly, in our experiments the cytotoxicity of tetranor-DHLA in the murine fibroblast cell line was, on average, 30% greater compared with BMTBA. The data obtained for human physiological fibroblasts showed no significant differences in the cytotoxicity of tetranor-DHLA and BMTBA.

Previous reports pointed out the ability of LA to exhibit antiproliferative activity towards tumor cells and revealed that the anticancer mechanisms of LA and DHLA and its derivatives involves cell cycle arrest and induction of apoptosis in tumor cells (CASCIARI et al., 2001, YAMASAKI et al., 2009). The present study supports these findings because EC_{80} values measured for the hepatoma cell line were almost two times lower for all the tested compounds than the values measured for physiological cells, for both the tested species.

Biological effectiveness of LA and its derivatives and anticancer activity may be related to their ability to modify the oxidation-reduction status of exposed cells (HAN et al., 1997; CASCIARI et al., 2001). In our previous studies antioxidant activity of the tested compounds was determined *in vitro* using two independent methods: ferric reducing/ antioxidant power assay (FRAP) and the influence on lipid peroxidation (determination of thiobarbituric acid-reactive substances, TBARS) (DUDEK et al., 2014). The performed experiments showed that LA and tetrano-DHLA had strong antioxidant properties. It corresponds with the present findings and it can be speculated that the antiproliferative action of these compounds towards hepatoma cells may be attributed to specific regulation of oxidative stress in cancer cells. At the same time, BMTBA does not exhibit any significant antioxidant potency, therefore probably additional mechanisms are involved in the antitumor performance of this compound.

In conclusion, the results of the present study revealed differences in cytotoxicity of LA, tetrano-DHLA and BMTBA, depending on the compound structure. The effect was species-related and the antiproliferative action of the tested drugs was explicit in the hepatoma cell line. LA is considered as a promising chemopreventive agent and we hope that the obtained data will contribute to successful design of new derivatives and to a better understanding of the role which particular compounds play in the living system.

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