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Original Research Article

Cytotoxic effect of Br-containing oxaphosphole on human melanoma cell line and pathogenic and symbiotic bacteria

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Keywords

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A B S T R A C T

Mitodepressive, clastogenic and apoptotic-like effects of Br-oxph (4-bromo-N,N-diethyl-5,5-dimethyl-2,5-dihydro-1,2-oxaphosphol-2-amine2-oxide) on plant and animal test-systems had been established. Br-oxph exerted different inhibitory effect on different cancer cells *in vitro*. The effects of Br-oxph on prokaryotic cells have not been studied yet. The present study was aimed to assess the cytotoxic effects Br-oxph on human melanoma cell line and on pathogenic and symbiotic bacteria. *In vitro* cytotoxicity assay: Morphology of human melanoma cell line A 2058 treated with Br-oxph (0.5, 1 and 2 mg/ml, for 24 and 48 hours) was analyzed under a light microscope. Antimicrobial test: *Staphylococus aureus* 745, *Enterobacter aerogenes* 3691 and *Lactobacillus acidophilus* were treated for 48 hours with Br-oxph (0.5 mg/ml, 1 mg/ml and 2 mg/ml), Sefpotec (10 mg/ml) and Biseptol (250 mg/ml). The antibacterial activity was assayed by the well diffusion method.Treatment with Br-oxph had no effect on growth of human melanoma cell line. Br-oxph (1 mg/ml for 48 hours) notably inhibited growth of pathogenic *S. aureus* and *E. aerogenes* and had no activity against symbiotic *L. acidophilus*. Biseptol and Sefpotec had a slight inhibitory effect against all bacteria tested. The present study indicated significant antibacterial activity of Br-oxph on tested pathogenic bacteria and lack of activity on symbiotic bacterium. This beneficial differentiation is in accordance with different cytotoxic influence of Br-oxph on different eukaryotic cells types. The data obtained in present study indicates Br-oxph as a potential candidate for drug development.

Introduction

Br-oxph (4-bromo-N,N-diethyl-5,5-dimethyl-2,5-dihydro-1,2-oxaphosphol-2-amine2-oxide) (Angelov and Enchev, 1987) belongs to the family of heterocyclic organophosphorus compounds.

Oxaphospholes possess biological activity, which is not well studied. Br-oxph is a structural analogue to compounds used as antiviral drugs (Tam *et al.*, 2000; Kamiya, 2003; Pescovitz, 2008; Okano, 2009) and

antitumor agents (Kanno *et al.,* 2009; Sharabi and Haran-Ghera, 2011).

In previous study *in vivo* we established mitodepressive and clastogenic effects of Br-oxph on plant and animal test-systems using light microscopy and atomic force microscopy (Kalcheva *et al.,* 2009a; Koleva *et al.,* 2013). Another sign of cytotoxicity of Br-oxph was apoptotic-like effect. Using conventional light microscopy we observed nuclear fragmentation and condensation in mice bone marrow cells after treatment with Br-oxph (Kalcheva *et al.,* 2009a; Kalcheva *et al.,* 2009b). The analysis via AFM (Koleva *et al.,* 2013) also confirmed morphology changes in treated with Br-oxph cells as described by others in apoptotic nuclei (Kam and Ferch, 2000; Gorneva *et al.,* 2005; Pelzel *et al*., 2010).

Apoptosis is a key point in therapeutical effects of anticancerous drugs (Bacsó *et al.,* 2000). Taking into account clastogenic and apoptotic effects of Br-oxph *in vivo* we evaluated growth inhibition potential *in vitro*. Br-oxph (1.8 mg/ml, for 3 hours) showed cytotoxic and apoptotic activity *in vitro* on lung carcinoma cell line SK-MES-1 (Koleva *et al.,* 2014). These results suggest necessity of further studies about possible application of Br-oxph as anticancerogenous compound. One of the greatest problems is the selective killing of different type cancer cells. The preliminary results revealed that inhibitory effect of Br-oxph on human hepatoma cell line SK-HEP-1 (2 mg/ml) was much stronger than those observed in SK-MES-1 cell line. These results suggest different cytotoxic influence of Br-oxph on different type cancer cells (Koleva *et al.,* 2014).

Our studies, listed above, evaluated influence of Br-oxph on eukaryotic test-objects. The effects of Br-oxph on prokaryotic cells have not been studied yet. Microorganisms affect the well being of people in a great many ways (Chaudhry *et al*., 2007). Antibiotics have been widely used for many years against bacteria that caused infectious diseases. Nowadays antimicrobial resistance is a threat to mankind (Saeed *et al*., 2007). Therefore, there is an urgent need to discover new compounds possessing potent antimicrobial activities (Mahalel, 2012). It must be noticed that antibiotics destroyed not only harmful, but also beneficial bacteria. The human gastrointestinal tract microbiota consists of a large number of bacteria involved in different physiological functions (Martín *et al*., 2013). The beneficial effects of antibiotics must be discussed taking into account their influence on intestinal microecology and the microenvironment (Huang *et al*., 2012). Antibiotic-associated diarrhea occurs in association with the administration of antibiotics (Bartlett, 1992; Gilbert, 1994). After antimicrobial therapy patients are instructed to take probiotics in order to recolonize the gastrointestinal tract (Rolfe, 2000). *L. acidophilus* is a beneficial bacteria that occurs naturally in the human and animal gastrointestinal tract. *L. acidophilus* strains could be used as probiotic (Ljungh and Wadström, 2006).

The present study was aimed to assess the cytotoxic effects Br-oxph on human melanoma cell line and on pathogenic and symbiotic bacteria.

Materials and Methods

Chemicals and reagents

Br-oxph was synthesized in the Laboratory of Organic Chemistry of the University of Shumen (Bulgaria) (Angelov and Enchev, 1987). Stock solutions of Br-oxph were freshly prepared in MEM. MEM growth media and fetal calf serum were purchased from PAA (Austria). Sefpotec and Biseptol

antibiotics were purchased from a pharmacy.

Cell lines and culture conditions

The human amelanotic melanoma cell line A 2058 was obtained from Medical university of Plovdiv (Bulgaria). The cells were maintained as adherent in controlled environment: MEM medium, supplemented by 10% heat-inactivated fetal calf serum, in incubator at 37ºC, 5% CO_2 and humidified atmosphere. In order to keep cells in log phase, the cultures were refed with fresh medium two or three times/week.

***In vitro* cytotoxicity assay (dose-response relationship)**

Exponentially growing cells were seeded in dishes (55 mm in diameter), at a density of 2×10^4 cells per ml in 3 ml. Time of treatment was 24 and 48 hours. At the end of incubation time the cell monolayer was stained for 20 min with 2% Giemsa solution. Morphology of stained cells was analyzed under a light microscope (160X). Two replications of each treatment were done.

Antimicrobial test

Chemicals and reagents

Media used: Nutrient agar (Biolife 272-20128, Milano, Italia) was the medium used as the growth medium for the microbes. The comersial probiotic strain cultivated in media of MRS (de Mann Rogosa Sharpe) (Biolife 4017292 Milano, Italia).

Compound tested: The solutions of Br-oxph (0.5 mg/ml, 1 mg/ml and 2 mg/ml), Sefpotec (10 mg/ml) and Biseptol (250 mg/ml) were freshly prepared in distilled water.

Test organisms: *Staphylococus aureus* 745 and *Enterobacter aerogenes* 3691 were obtained from Collection of Department of General and Applied Microbiology, Sofia University. The probiotic strain used – *Lactobacillus acidophilus* was obtained from commercial probiotic product purchased from pharmacies. All the isolates were checked for purity and maintained in slants of Nutrient agar.

Assay for antimicrobial activity

Antimicrobial assay was performed by the well diffusion method using soft 0.8% agar. Agar medium was added to sterile Petri dishes seeded with 100 µl of each test bacterial strains. Wells of equal distance were dug on the seeded plates. Each well was filled up with 100 µl of the solutions tested. After adjusting the pH at 6.5 by NaOH, the activity of the plant extracts was checked. The plates were incubated at 37°C for 48 hours. The antibacterial activity was assayed by measuring the diameter of the inhibition zone formed around the well. All experiments were performed in triplicate.

Results and Discussion

In vitro cytotoxicity assay

Our previous study (Koleva *et al.*, 2014) suggests different cytotoxic influence of Br-oxph on different type cancer cells: Br-oxph (1.8 mg/ml and 3.6 mg/ml, for 3 hours) showed cytotoxic and apoptotic activity in vitro on *lung carcinoma cell line* SK-MES-1; the inhibitory effect of Br-oxph on human hepatoma cell line SK-HEP-1 (2 mg/ml) was much stronger than those observed in SK-MES-1 cell line. For this reason the goal of present study was to examine the antiproliferative and apoptotic activity of Br-oxph in different cancerous cell line.

Treatment with Br-oxph (0.5, 1 and 2 mg/ml, for 24 and 48 hours) had no effect

on growth of human melanoma cell line. As can be seen in Figure 1, the untreated cells exhibited normal shapes, with clear outline. There were no morphological changes (shape, size and nuclear structure) in treated cells. These results confirmed our previous observation of different cytotoxic influence of Br-oxph on different type cancer cells (Koleva *et al.,* 2014).

Antibacterial test

In present study effects of Br-oxph on two pathogenic bacteria (Gram-positive and Gram-negative *S. aureus* 745 and *E. aerogenes* 3691) and symbiotic *L. acidophilus* were evaluated. The effects were compared with two widely used antibiotics: Biseptol and Sefpotec. Both antibiotics used are known to possess broad spectrum antibacterial activity against both gram-positive and gram-negative organisms. The sensitivities of the test organisms to infusions were indicated by clear zone around the wells (Figure 2). Br-oxph at concentration 1 mg/ml for 48 hours notably inhibited growth of *S. aureus* (27.98 mm mean zone of inhibition +/-4.38 SD) and *E. aerogenes* (25.37 mm mean zone of inhibition +/-2.85 SD). On the contrary, Br-oxph had no activity against *L. acidophilus* (9.24 mm mean zone of inhibition +/-0.33 SD).

Biseptol at concentration 250 μg/ml (normally used by prescription) for 48 hours had mean zone of inhibition of 10.79 mm (+/-0.76 SD) against *S. aureus* and 11.47 mm (+/-1.39 SD) against *E. aerogenes*. Biseptol also inhibited growth of *L. acidophilus* – mean zone of inhibition was 12.38 mm (+/-0.11 SD).

The negative effect of Sefpotec at concentration tested (250 μg/ml, normally used by prescription) was similar to those exerted by Biseptol – mean zones of inhibition were respectively 13.57 mm (+/-0.45 SD) and 14.73 mm (+/-0.03 SD). Sefpotec also influence negatively *L. acidophilus,* but to a lesser extend as compared to pathogenic bacteria – mean zone of inhibition was 11.01 mm (+/-0.03 SD).

The chemistry of organophosphorus compounds is a subject of increasing interest. A great number of new compounds with different structures and respectively, with different properties have been synthesised (Smee and Reist, 1996; Brel, 2008; Leblond *et al.*, 2002). The results of present study revealed that Br-oxph possess antibacterial activity against *Staphylococcus aureus* 745 and *Enterobacter aerogenes* 3691. The growth inhibitory activity of Br-oxph against pathogenic Gram-positive and Gram-negative bacteria tested was much stronger in comparison of activity of antibiotics Biseptol and Sefpotec. Nowadays antibiotic resistance has reached alarming levels in many parts of the world (WHO, 2014). Antibiotics are critical in the fight against bacterial infectious diseases leading to necessity of new antimicrobial compounds.

The inhibitory effect against bacterial species tested indicates Br-oxph as a potential candidate for drug development for the treatment of infectious diseases caused by these pathogens. These results raise requirement of further studies of antimicrobial activity of compound tested.
No inhibitory effect of Br-oxph on *L. acidophilus* was observed. It must be noticed that antibiotics used in present study revealed activity against *L. acidophilus*. The different effect of Br-oxph on bacteria tested is in accordance with different activity of Br-oxph observed in experiments with eukaryotic cells *in vitro*. This beneficial

differentiation leads to necessity of further studies with other valuable symbiotic bacteria.

It must be noticed that existence of antimicrobial effect was observed even 3 hours after treatment. This observation is in accordance with data that Br-oxph appeared to influence different cellular processes in eukaryotic cells after 3 h treatment period (Kalcheva *et al.*, 2009a; Kalcheva *et al.*, 2009b; Koleva *et al.*, 2014).

Figure.1 Human melanoma cell line A 2058: untreated control (A); after treatment for 48 h with Br-oxph at concentration 0.5 mg/ml (B), 1 mg/ml (C) and 2 mg/ml (D)

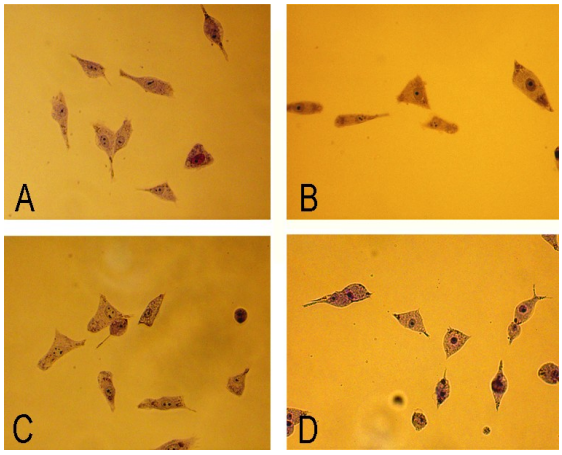
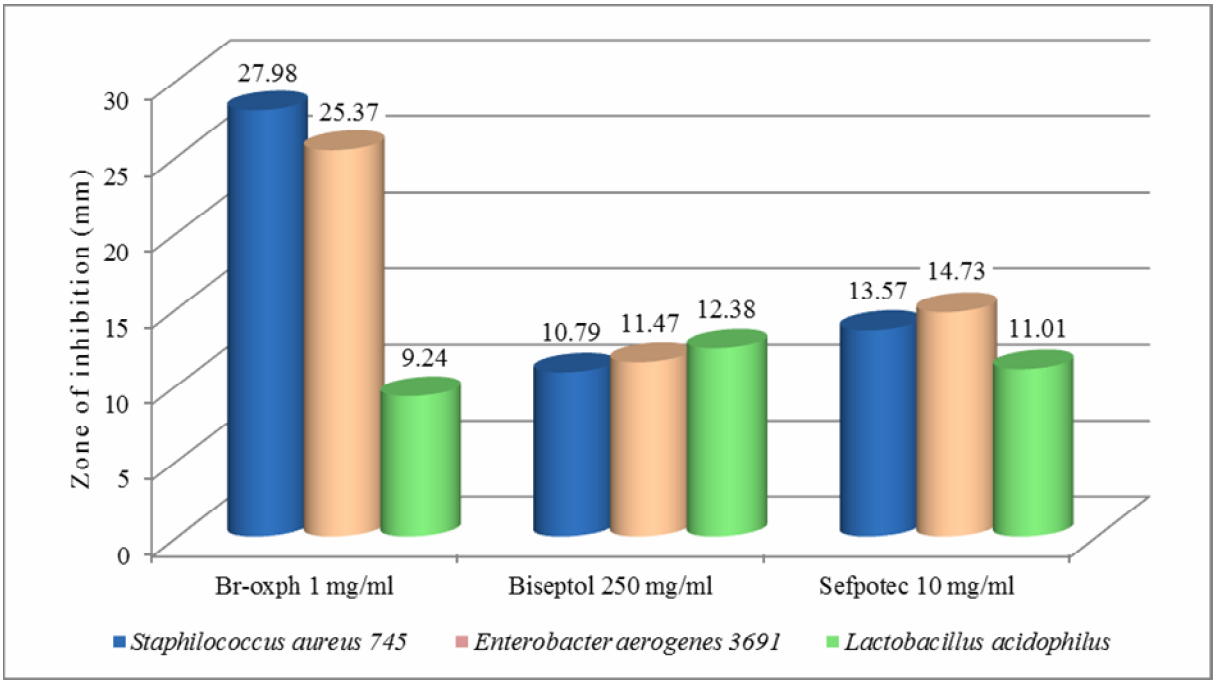


Figure.2 Antibacterial effect of Br-oxph (1 mg/ml), Biseptol (250 mg/ml) and Sefpotec (10 mg/ml) for 48 hours



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