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Original scientific paper

INHIBITION OF GROWTH AND INDUCTION OF APOPTOSIS IN HUMAN LUNG CANCER CELLS BY BR-OXPH

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The study was aimed at evaluating apoptotic potential of Br-oxph (*4-bromo-N,N-diethyl-5,5-dimethyl-2,5-dihydro-1,2-oxaphosphol-2-amine 2-oxide*) *in vitro*. The dose response effect of Br-oxph (dose range 1-3 mg/ml, for 48 h) on SK-MES-1 cells viability was determined by means of WST-1 cell proliferation assay. The half maximal inhibitory concentration (IC50) value was determined – 1.8 mg/ml. The ability of the compound tested to induce apoptosis was tested by ELISA to detect cellular DNA fragmentation. We provided a quantitative assessment of the apoptotic potential of Br-oxph in human lung carcinoma cells at concentrations corresponding to IC50 and 2xIC50 for 3 hours. Treatment with 2xIC50 significantly increased the amount of cytoplasmic DNA-fragments. Results obtained from the present study confirm that Br-oxph target the cancerous cells towards apoptosis.

Key words: Apoptosis, Br-oxph, DNA fragmentation, cancer cell line, cell viability

INTRODUCTION

4-bromo-N,N-diethyl-5,5-dimethyl-2,5-dihydro-1,2-oxaphosphol-2-amine2-oxide (Br-oxph) belongs to the family of heterocyclic compounds, containing phosphorus and oxygen atoms in the ring – oxaphospholes (Figure 1). Oxaphospholes possess biological activity, which is not well studied. Br-oxph is a structural analogue to furanoses. Furanoses are abundant constituents of natural products, including nucleic acids. Nucleoside-analogue derivatives have received wide attention as antiviral drugs (TAM *et al.,* 2000; KAMIYA, 2003; PESCOVITZ, 2008; OKANO, 2009). Another compound with structural similarity to Br-oxph is cyclophosphamide. Cyclophosphamide belongs to a class of drugs known as alkylating agents, which have been used to treat some types of cancer (KANNO *et al.,* 2009; SHARABI and HARAN-GHERA, 2011).

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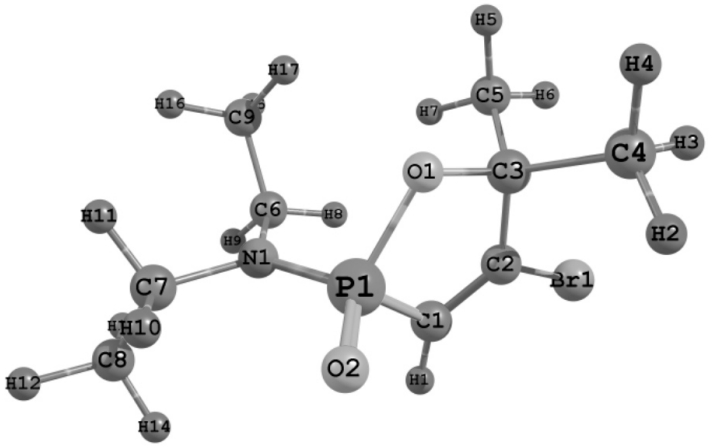

Figure 1. Molecular models are optimized with Firefly QC package (GRANOVSKY, Firefly version 7.1.G), which is partially based on the GAMESS (US) (SCHMIDT *et al*., 1993) source code. Provided to us by A. Patleeva.

Organophosphorus compounds are widely used as pesticides (RICHARDS *et al.,* 2000; CASIDA and DURKIN, 2013). In previous study *in vivo* we established cytotoxic and genotoxic effects of Br-oxph at low doses in plant and animal test-systems (KALCHEVA *et al.,* 2009 a). Clastogenic effect of Br-oxph at very low dose ($2.82x10^{-9}$ μg/kg) was confirmed at the ultrastructural level using atomic force microscopy (KOLEVA *et al.,* 2013). These results revealed that possible application of Br-oxph as agrochemical could affect negatively environmental and human health.

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.,* 2009 a; KALCHEVA *et al.,* 2009 b). The analysis via AFM (KOLEVA *et al.,* 2013) also confirmed morphology changes described in apoptotic nuclei by others (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). Most chemotherapy drugs are genotoxic (TURINETTO *et al.,* 2009) and effectiveness of chemotherapy depends on inhibition of cell proliferation and/or triggering of apoptosis in tumor cells (JESSEN *et al.*, 2005; SHENOY *et al.,* 2007; RASTOGI *et al.*, 2009).

Clastogenic and apoptotic effects of Br-oxph *in vivo* raise the hypothesis of another possible application of compound studied – growth inhibition of tumor cells. The objective of present study was to determine the apoptotic potential of Br-oxph in human cancerous cells lines.

MATERIALS AND METHODS

Chemicals and reagents

MEM growth media and fetal calf serum were purchased from PAA (Austria). Premixed WST-1 Cell Proliferation Reagent and Cellular DNA Fragmentation ELISA kit were purchased from Roche Applied Science.

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 (1 mg/ml, 1.5 mg/ml, 2 mg/ml, 2.2 mg/ml, 2.4 mg/ml, 2.6 mg/ml, 2.8 mg/ml and 3 mg/ml) were freshly prepared in MEM.

Cell lines and culture conditions

The SK-MES-1 was obtained from National Bank for industrial Microorganisms and Cell Cultures (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)

Cell viability was assessed using Premixed WST-1 Cell Proliferation Reagent. The assay principle is based upon the reduction of the tetrazolium salt WST-1 to formazan in the mitochondria of living cells. Exponentially growing cells were seeded in 96-well flat-bottomed microplates (100 µL/well) at a density of $2x10^4$ cells per ml. Time of treatment was 48 hours. Four hours before the end of incubation time, cell proliferating reagent WST-1 (10 µl/ well) was added to the culture media. Microplates were further incubated for 4 hours at 37°C. The absorbance of formazan product was quantitated at 450 nm using an ELISA reader. The cell survival fractions were calculated as a percentage of the untreated control (untreated control = 100%). Dose response curves were created by plotting the percent of viable cells versus the test concentrations. Concentration of Br-oxph showing 50% reduction in cell viability (half maximal inhibitory concentration value, IC50) was then calculated.

DNA fragmentation analysis

The ability of Br-oxph to induce apoptosis was studied using Cellular DNA Fragmentation ELISA kit as per supplier's instructions. Briefly, the cell number was adjusted to $3x10^5$ cells/ml in the culture medium and BrdU labeling solution was added to a final concentration of 10 µM. After BrdU labelling for 4 h cells were treated with IC50 (1.8 mg/ml) and 2xIC50 (3.6 mg/ml) Br-oxph for 3 h. Cells were lysed and centrifuged at 250 *g* for 10 minutes. Each well of anti-DNA antibody coated microtiter plate were added with 100 µl of the supernatant. Further analysis was provided by ELISA, using the instructions recommended by the manufacturer.

Data processing and statistics

The cytotoxicity assays were carried out in 9 replicate wells. Tthe apoptosis induction evaluation was conducted in 6 replicate wells. The values for each concentration tested represent the average (mean ± SD). Student's t-test was performed with $P \leq 0.05$ taken as significance level.

RESULTS AND DISCUSSION

Experiments aimed to determine the dose response effect of Br-oxph (dose range 1-3 mg/ml, for 48 h) on SK-MES-1 cells proliferation/viability were carried out by means of WST-1 cell proliferation assay. The results revealed concentration-dependent and statistically significant ($P \leq 0.001$) inhibition effect of Br-oxph at concentrations tested (Figure 2). As can be seen,

treatment with 1 mg/ml Br-oxph showed about 24% reduction in cell viability. After addition of 1.5 mg/ml Br-oxph viability was reduced by 40%. The inhibitory effect of the compound tested increased at dose range 2-2.4 mg/ml (reduction in cell viability about 57%). The percent of viable cells decreased at higher concentrations tested: after treatment with 3 mg/ml Br-oxph only 26% of cells survived.

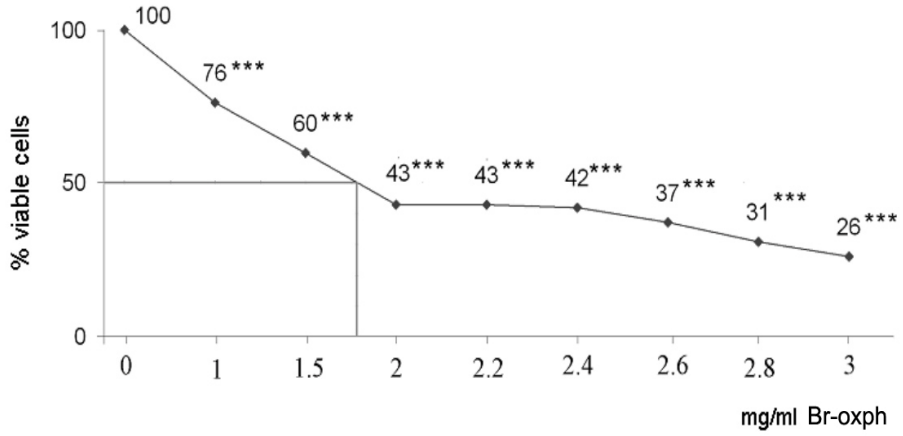

Figure 2. Effect of Br-oxph on cell proliferation/cell viability of lung carcinoma cell line (SK-MES-1). *** P≤0.001.

From the curve based on the obtained values plotted as percentage of viable cells against Br-oxph test concentrations the IC50 were obtained – 1.8 mg/ml.

As a next step, we checked the ability of Br-oxph to induce apoptosis, a process that removes highly damaged cells. A hallmark of apoptosis is fragmentation of the DNA and DNA-histone complex are released to the cytoplasm (AU and WIENTJES, 1999; BACSÓ *et al.,* 2000). We provided a quantitative assessment of the apoptotic potential of Br-oxph at concentrations corresponding to IC50 and 2xIC50. Consistent with other studies (BACSÓ *et al.,* 2000; KRÜGER *et al.,* 2009; MUTEE *et al.,* 2012) the duration of the treatment was 3 h.

The effect of treatment with Br-oxph (1.8 mg/ml and 3.6 mg/ml) for 3 hours on internucleosomal DNA fragmentation in lung carcinoma cells is shown in Figure 3. The apoptotic effect of compound tested is dose dependent. After treatment with Br-oxph at concentration 1.8 mg/ml (IC50) we observed only slight elevation of the DNA-fragments in cytoplasm – by 11% in comparison with the control. Treatment with 2xIC50 significantly increased the amount of DNA-fragments – by 33% as compared to the control. This finding is in accordance with our previous results revealing clastogenic activity of compound tested in mice bone marrow cells 3 h after the treatment (KALCHEVA *et al.,* 2009 a; KALCHEVA *et al.,* 2009 b).

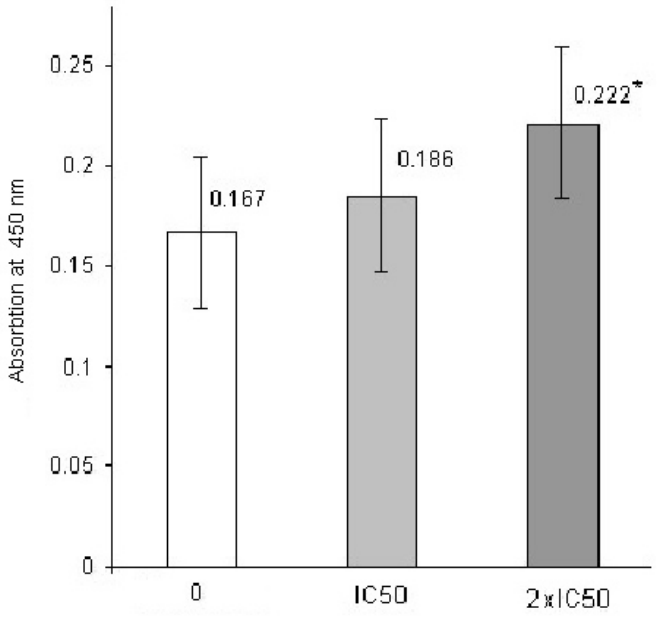

Figure 3. Apoptosis induction by Br-oxph in lung carcinoma cell line SK-MES-1 as determined by ELISA for DNA Fragmentation. * P≤0.05.

The results of our studies revealed clastogenic effects of Br-oxph on cellular DNA. According to BOLDERSON *et al.* (2009) the potential ways to manipulate DNA damage response could impact enormously future medical science. The established antiproliferative and apoptotic activity of Br-oxph raises the possibility of using the compound tested in treatment of some human cancers. DNA double-strand breaks (DSBs) are potentially lethal events for the cell (HALAZONETIS *et al*., 2008; ROOS and KAINA, 2013). The answer of the cell is DNA repair and/or cell cycle blocking. In occasion of failure of the repair machinery to rejoin the breaks, the programmed cell death was triggered (LIM *et al*., 2009; SHI and OBERDOERFFER, 2012). Programmed cell death (apoptosis) is also a key mechanism by which anticancer therapies exert their therapeutic effects (BACSO *et al.,* 2000). The transcription factor p53 plays a key role in both types of cell answer (HOSAKO *et al.,* 2007; OZAKI and NAKAGAWARA, 2011; REINHARDT and SCHUMACHER, 2012; XU *et al.,* 2013). Taking into account apoptotic and clastogenic activity of Br-oxph, we can speculate that the compound tested induces apoptosis by mechanism involving p53 (Figure 4). Under normal conditions, p53 is expressed at an extremely low level, which is caused by proteasomal degradation mediated largely by ligase MDM2 (OZAKI and NAKAGAWARA, 2011). ATM-kinase (Ataxia-Telangiectasia Mutated) is the predominant kinase responsible for the activation of multiple cell cycle checkpoints following DSB induction (BOLDERSON *et al.,* 2009). ATM phosphorylates Chk2 (checkpoint kinase-2) after the formation

of DSBs. In turn, Chk2 phosphorylate the transcription factor p53. This prevents its proteosomal degradation and p53 transcriptionally regulates pro-apoptotic genes such Bax, Puma, Noxa (OZAKI and NAKAGAWARA, 2011; SONG *et al.,* 2011; REINHARDT and SCHUMACHER, 2012).

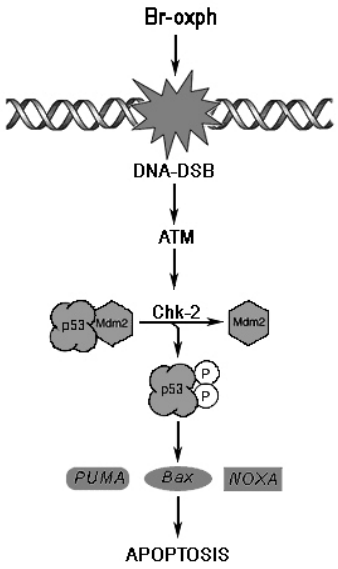

Figure 4. A possible mechanism of triggering apoptosis after treatment with Br-oxph.

There is a clear need for new and effective drugs for different kind of malignancies (BURGER and FIEBIG, 2004; ABU-SURRAH and KETTUNEN, 2006). Drug development is complicated, time-consuming, and costly process. ZHOU and WONG (2006) described a recently emerged promising solution to improve the quality of decisionmaking in drug development. According to authors, cell-based assays could be used for the development of new drugs starting from primary screening to *in vitro* toxicology. Using such a strategy in present study we established antiproliferative and apoptotic activity of Br-oxph, suggesting possible antitumor activity.

One of the greatest problems is the selective killing of different type cancer cells. For this reason the other goal of our study was to examine the antiproliferative and apoptotic activity of Br-oxph in different cancerous cell line. We provided WST-1 Cell Proliferation Assay using Human hepatoma cell line SK-HEP-1. The preliminary results revealed that inhibitory effect of Br-oxph on SK-HEP-1 cells was much stronger than those observed in SK-MES-1 cell line: after treatment with 2 mg/ml survived cells were 7% in comparison with control (data not presented). These results suggest selective cytotoxic influence of Br-oxph on different type cancer cells.

CONCLUSION

The data obtained in present study showed cytotoxic and apoptotic activity of Br-oxph (1.8 mg/ml and 3.6 mg/ml) for 3 hours on lung carcinoma cell line SK-MES-1. These results suggest necessity of further studies about possible application of Br-oxph as anticancerogenous compound.

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SPREČAVANJE ŠIRENJA I INDUKCIJA APOPTOZE U LJUDSKIM ĆELIJAMA RAKA PLUĆA POMOĆU BR-OXPH

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Izvod

Studija je imala cilj procenu apoptotskog potencijala Br- oxph (*4-bromo-N,N-diethyl-5,5-dimethyl-2,5-dihydro-1,2-oxaphosphol-2-amine 2-oxide*) *in vitro*. Efekat doznog odgovora Br-oxph (veličina doze 1-3 mg/ml, za 48 sati) na vitalnost SK-MES-1 ćelija je određen pomoću VST -1 testa proliferacije ćelija. Vrednost polu – maksimalne inhibitorne koncentracije (IC50) određena je na 1.8 mg/ml. Sposobnost testiranog jedinjenja da indukuje apoptozu je ispitana pomoću ELISA testa sa ciljem da se detektuje ćelijska DNK fragmentacija. Obezbedili smo kvantativnu procenu apoptotskog potencijala Br-oxph kod ćelija ljudskog karcinoma pluća u koncentracijama koje odgovaraju IC 50 i 2 x IC50 za 3 sata. Lečenje pomoću 2 x IC 50 znatno je povećalo količinu citoplazmatskih fragmenata DNK. Rezultati dobijeni iz ove studije da Br-oxph ciljno napadaju kancerogene ćelije u smeru apoptoze.

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