Exp Oncol 2011
33, 2, 107–109

SHORT COMMUNICATIONS

Experimental ONCOLOGY

PHOTOLON ENHANCEMENT OF ULTRASOUND CYTOTOXICITY

*D.A. Tserkovsky, E.N. Alexandrova, Yu. P. Istomin**

N.N. Alexandrov National Cancer Center of Belarus, 223040 Minsk, Belarus

Objective: **To evaluate the impact of Photolon on cytostatic and cytotoxic effects of therapeutic range ultrasound in C6 glioma cells.** ***Methods:*** **C6 glioma cells in suspension or monolayer cell culture were exposed to ultrasound (880 kHz, 0.2–0.7 W/cm^2) in the presence or absence of Photolon at the concentration of 1 μg/ml in the culture medium, and then cell viability was evaluated.** ***Results:*** **Photolon increased the cytotoxic effect of ultrasound by 1.5–2.3-fold but had no effect on its cytostatic activity.** ***Conclusion:*** **Photolon produces a pronounced sonosensitizing effect on glioma C6 cells and is a promising drug for sonodynamic treatment of malignant tumors.**

Key Words: **photolon, ultrasound, C6 glioma cells.**

Ultrasound is widely used in medicine for diagnosis and treatment of many diseases. The use of ultrasound for therapeutic purposes is based on the specific nature of the interaction between ultrasound and biological tissues. Structural and functional changes in biological tissues exposed to ultrasound depend on the parameters of ultrasonic radiation: its frequency, intensity and duration of exposure. High-frequency ultrasound (1.0–31.5 MHz) is characterized by predominant thermal effect, whereas ultrasound with low frequency produces cavitation. Physiotherapeutic practice uses ultrasonic waves with 800 to 3000 kHz frequency and up to 3 W/cm^2 intensity.

Investigation of biological effects of ultrasonic waves with different frequency, intensity and duration of action has shown that ultrasound exerts antitumor activity. In experimental studies, ultrasound of therapeutic range was found to cause growth inhibition and death of human tumor cells: leukemia K562, HL-60, KG1a and Nalm-6 cells [1, 2], breast adenocarcinoma MCF-7 cells, ovarian carcinoma SC-OV-3 [3], epidermoid cancer [4], cervical carcinoma HeLa cells [5–7], colon carcinoma HT-29 cells, gingival carcinoma Ca9-22 cells [7], glioma C6 cells [8, 9], as well as implanted rat tumors: Zeidel hepatoma, M-1 sarcoma [4], RS-1 cholangiocellular carcinoma [10], C6 glioma [11]. High-intensity (3 W/cm^2 to 10^4 W/cm^2) focused ultrasound with 1–10 MHz frequency, the absorption of which results in a focal temperature of 40–100 °C, is used in oncology for non-invasive surgery of deep-seated tumors, as well as for induction of local hyperthermia.

Moreover, exposure to ultrasound of a specific frequency range, intensity and duration was found to improve the efficacy of anticancer drugs both *in vitro* and *in vivo* without causing direct destruction of tumor cells, which is attributed to increased permeability of cell membranes for cytostatics [10, 12]. The potential gain in radiosensitivity of tumor cells due to ultrasound pretreatment was also demonstrated [13, 14].

A new trend, sonodynamic therapy of malignant tumors is being developed, which is based on tumor destruction as a result of synergetic enhancement of ultrasound effect by a sonosensitizer drug. The terms "sonodynamic therapy" and "sonosensitizer" were proposed by Umemura *et al*.(1990) by analogy with the terms of photodynamic therapy, as the classical photosensitizer hematoporphyrin had also been shown to possess sonosensitizing properties [15]. The mechanism of sonodynamic therapy seems to be associated with the damage of cell membranes. The advantage of sonodynamic therapy over photodynamic one is its ability to focus ultrasound in the area of deep-seated tumors.

In experimental models *in vitro* on tumor cells and *in vivo* on transplanted tumors in laboratory animals, the sonosensitizing properties of porphyrin photosensitizers and a number of other compounds were studied [12]. Such photosensitizers as hematoporphyrin, Photofrin, Photofrin II, mesoporfirin, protoporphyrin, protoporphyrin IX, TPPS, ATX-70, ATX-S10, pheophorbide-a, AlPcS4, chlorine PAD-S31 [15], methylene blue [16], erythrosine, rhodamine [17], NPe6 [18], rose bengal [11], 5-ALA [19], chlorin e6 [20] have been shown to enhance ultrasound cytotoxic effect on tumor cells and implanted tumors. The results of the experimental studies suggest that sonodynamic therapy is a promising treatment modality for malignant tumors.

The purpose of this study was to investigate a sonodynamic activity of the original Belarusian photosensitizer Photolon.

The study was conducted on C6 rat glioma cell line (Russian Collection of Cell Cultures, Cytology Institute of Russian Academy of Sciences, St. Petersburg). The cells were cultured in DMEM culture medium supplemented with 10% fetal calf serum.

Exponentially growing cells as a monolayer or suspension culture were used for the experiments. To suspend the cells, the monolayer was detached from the culture flasks with 0.02% versen.

The drug Photolon (RUE Belmedpreparaty, Belarus) was diluted with saline just before the use.

The cells were sonicated with ultrasound of 0.2, 0.4 or 0.7 W/cm^2 intensity at room temperature for

Received: March 5, 2011.

*Correspondence: E-mail: istomin06@mail.ru

Abbreviations used: II_{50} — ultrasound intensity required for a 50% inhibition of cell proliferation; IL_{50} — ultrasound intensity required for a 50% cell death; ER — enhancement ratio.

60 s, using UST-1.040 apparatus for ultrasonic therapy (880 kHz, EMA Plant, Russia) in the continuous ultrasonic generation mode. The emitter was placed directly into the culture medium.

Photolon (1 μg/ml) was added to the culture medium 5 min before sonication of the cell suspension or 2 h before sonication of the cell monolayer (for maximum accumulation of Photolon in the cells).

The cytotoxic effect of ultrasound on C6 glioma cells in the suspension was assessed by trypan blue dye exclusion test 5 min after the exposure.

To evaluate the cytostatic effect of ultrasound, the monolayer culture growth of C6 glioma cells after the treatment was studied. 24 h after sonication, the monolayer was detached from the culture flasks with 0.02% versen, and viable (trypan blue unstained) cells were counted in a hemocytometer. The increase in the cell number compared to the control value was calculated from: $(N_t-N_o)/(N_c-N_o)$ if N_t was greater than or equal to N_o, or $(N_t-N_o)/N_o$, if N_t was less than N_o (N_t — cell number in the treated culture; N_c — cell number in the control culture; N_o — initial cell number before the treatment).

The values of II_{50}, ultrasound intensity required for a 50% inhibition of cell proliferation, and LI_{50}, ultrasound intensity required for a 50% cell death, were calculated using regression analysis of the data obtained. The enhancement ratio (ER) for inhibition of cell proliferation or cell killing was calculated as II_{50} or LI_{50} for ultrasound without Photolon divided by II_{50} or LI_{50} for ultrasound with Photolon.

The values obtained were processed using standard statistical methods of Origin 7.0.

To assess the cytotoxic effect of ultrasound, C6 glioma cell suspension (785×10^3 cells/ml) was sonicated at 0.2, 0.4 or 0.7 W/cm² ultrasound intensity. Then the number of viable cells was calculated by trypan blue dye exclusion test.

Fig. 1, a, presents the number of viable C6 cells in suspension after the exposure to ultrasound alone or in combination with Photolon. The results suggest that sonication leads to a decrease in the number of viable cells. The cell death increased with ultrasound intensity; LI_{50}, ultrasound intensity required for a 50% cell death, was 0.51 W/cm².

Photolon addition to the cell suspension resulted in a significant enhancement of ultrasound cytotoxicity at 0.4 and 0.7 W/cm² intensity, with LI_{50} decreasing to 0.33 W/cm². The differences in cell number after the exposure to ultrasound alone and ultrasound with Photolon were statistically significant ($p < 0.01$). Thus, the gain in ultrasound cytotoxicity in the presence of Photolon was 1.5.

It should be noted that Photolon alone caused a decrease in the number of viable cells only by 16% compared to the control. The temperature of the culture medium during cell treatment with ultrasound did not rise.

The effect of varying intensity ultrasound on the growth of the monolayer culture of C6 glioma cells was assessed by the number of viable cells 24 h after the exposure.

The data presented in Fig. 1, b, suggest that sonication leads to growth inhibition of C6 glioma cells, the number of viable cells reducing with increased ultrasound intensity. Regression analysis of the data revealed that II_{50}, the ultrasound intensity needed for a 50% inhibition of cell proliferation, was 0.27 W/cm², and LI_{50}, the ultrasound intensity required for a 50% cell death, was 1.32 W/cm².

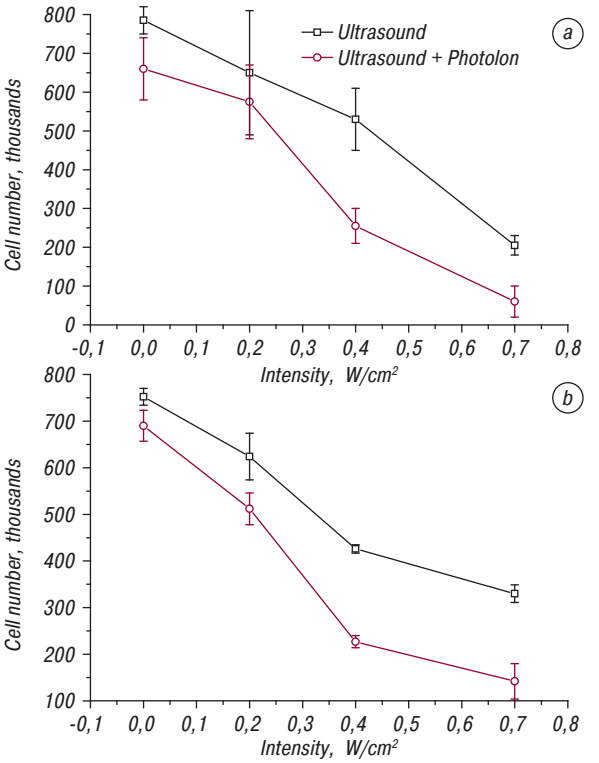

Fig. 1. Effect of ultrasound alone or in combination with Photolon on the number of viable C6 glioma cells in suspension (a) or in monolayer (b)

Photolon addition to the culture medium before sonication resulted in an enhanced effect of ultrasound on C6 cell monolayer, the number of viable cells after the exposure being statistically significantly lower ($p < 0.01$) than after ultrasound alone (Table). II_{50} was 0.28 W/cm², and LI_{50} was 0.58 W/cm². Thus, Photolon did not intensify growth inhibition of monolayer cell cultures after sonication. But cell death increased 2.3-fold when ultrasound was combined with Photolon compared to the effect of ultrasound alone. It should be noted that Photolon alone had almost no effect on the growth of C6 glioma cell cultures, the number of viable cells reducing by 8% compared to the control.

Table. Cytostatic and cytotoxic effect of ultrasound (W/cm²) on C6 cells

Treatment	Cell culture			
	Suspension		Monolayer	
	II_{50}	LI_{50}	II_{50}	LI_{50}
US	-	0.51±0.03	0.27±0.03	1.32±0.03
US+Photolon	-	0.33±0.03 (ER=1.5)	0.28±0.02 (ER=1.0)	0.58±0.02 (ER=2.3)

Photodynamic therapy with various photosensitizers including Photolon is currently used in multimodality treatment of malignant gliomas. The original photosensitizer Photolon, a complex of chlorin e6 salt

and polyvinylpyrrolidone, is selectively accumulated in tumor tissues, displays strong photodynamic activity and is registered as an agent for photodynamic therapy of malignant neoplasms. Photolon is able to penetrate the blood-brain barrier, and it may be used for intraoperative photodynamic therapy in patients with primary and metastatic brain tumors [21].

The feasibility of sonodynamic therapy of glial brain tumors is under study. Indeed, sonodynamic activity of hematoporphyrin [8, 9] and rose bengal [11] photosensitizers in experimental models of glioma *in vitro* and *in vivo* has been investigated. The authors have reported pronounced antitumor efficacy of ultrasound in combination with these photosensitizers in the treatment of experimental malignant gliomas.

The results of our study on C6 glioma cells suggest that Photolon also displays pronounced sonosensitizing activity and is a promising drug for sonodynamic treatment of gliomas.

REFERENCES

1. **Lagneaux L, de Meulenaer EC, Delforge A, *et al.*** Ultrasonic low-energy treatment: A novel approach to induce apoptosis in human leukemic cells. Exp Hematol 2002; **30:** 1293–301.

2. **Ashush H, Rozenszajn LA, Blas M, *et al.*** Apoptosis induction of human myeloid leukemic cells by ultrasound exposure. Cancer Res 2000; **60:** 1014–20.

3. **Sergeyeva NS, Sviridov IK, Nikolayev AL, *et al.*** Effect of different modes of low-frequency ultrasound on the survival of human tumor cells *in vitro*. Bull Exper Biol Med 2001; **131:** 331–4 (In Russian).

4. **Biryukov YuV, Sheremetyeva GF, *et al.*** Effect of low-frequency ultrasound on tumor cells in experimental models. Thorac Surg 1989; **3:** 64–9 (In Russian).

5. **Kaufman GE, Miller MW, Griffiths TD, *et al.*** Lysis and viability of cultured mammalian cells exposed to 1 MHz ultrasound. Ultrasound Med Biol 1977; **3:** 21–5.

6. **Hrazdira I, Skorpıkova J, Dolnıkova M.** Ultrasonically induced alterations of cultured tumour cells. Eur J Ultrasound 1998; **8:** 43–9.

7. **Fang HY, Tsai KC, Cheng WH, *et al.*** The effects of power on–off durations of pulsed ultrasound on the destruction of cancer cells. Int J Hyperthermia 2007; **23:** 371–80.

8. **Li J, Song D, Xu Y, *et al.*** *In vitro* study of haematoporphyrin monomethyl ether-mediated sonodynamic effects on C6 glioma cells. Neurol Sci 2008; **29:** 229–35.

9. **Dai S, Hu S, Wu C.** Apoptotic effect of sonodynamic therapy mediated by hematoporphyrin monomethyl ether on C6 glioma cells *in vitro*. Acta Neurochir 2009; **151:** 1655–61.

10. **Andronova NV, Filonenko DV, Bozhevolnov VE, *et al.*** Combination therapy of malignant tumors using local ultrasound treatment (experimental study). Rus Biother J 2005; **4:** 103–7 (In Russian).

11. **Nonaka M, Yamamoto M, Yoshino S, *et al.*** Sonodynamic therapy consisting of focused ultrasound and a photosensitizer causes a selective antitumor effect in a rat intracranial glioma model. Anticancer Res 2009; **29:** 943–50.

12. **Rosenthal I, Sostaric JZ, Riesz P.** Sonodynamic therapy––a review of the synergistic effects of drugs and ultrasound. Ultrasonics Sonochem 2004; **11:** 349–63.

13. **Martins BI, Raju MR, Hayes TL, *et al.*** Survival of cultured mammalian cells exposed to ultrasound. Rad Environm Biophys 1977; **14:** 243–50.

14. **Kobayashi H, Sakuma S.** Treatment of human superficial tumors by ionizing radiation combined with ultrasound. Nippon Igaku Hoshasen Gakkai Zasshi 1991; **51:** 149–54.

15. **Umemura S, Yumita N, Nishigaki R, *et al.*** Mechanism of cell damage by ultrasound in combination with hematoporphyrin. Jpn J Cancer Res 1990; **81:** 962–6.

16. **Zabolotny DI, Pukhlik SM, Piontkovskaya MB.** New applications of sonodynamic therapy in oncorhinology. Bul Otorhinolaryngol 2006; **3:** 23–6 (In Russian).

17. **Hiraoka W, Honda H, Feril LB, *et al.*** Comparison between sonodynamic effect and photodynamic effect with photosensitizers on free radical formation and cell killing. Ultrasonics Sonochem 2006; **13:** 535–42.

18. **Yumita N, Han Q-S, Kitazumi I, *et al.*** Sonodynamically-induced apoptosis, necrosis, and active oxygen generation by mono-l-aspartyl chlorin e6. Cancer Sci 2008; **99:** 166–72.

19. **He Y, Xia X, Xu C, *et al.*** 5-Aminolaevulinic acid enhances ultrasound-induced mitochondrial damage in K562 cells. Ultrasonics 2010; **50:** 777–81.

20. **Zheng R, Zhang W, Wang X, *et al.*** The sonodynamic effects of chlorin e6 on the proliferation of human lung adenocarcinoma cell SPCA-1. Chin J Lung Cancer 2010; **13:** 201–5.

21. **Trukhacheva TV, Shlyakhtin SV, Isakov GA, *et al.*** Photolon®, a new tool for photodynamic therapy. Review of the results of pharmaceutical, pharmacological and clinical trials. Minsk: RUE Belmedpreparaty 2009. 64 p (In Russian).