Diazepam enhances hypericin-induced photocytotoxicity and apoptosis in human glioblastoma cells*

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Glioblastoma multiforme (GBM) is neoplasm which is resistant to all currently used treatment modalities including surgery, radiation therapy and chemotherapy. Photodynamic therapy (PDT) has been suggested as a novel therapeutical approach to the treatment of malignant gliomas. Here, we attempted to enhance hypericin-induced photocytotoxicity and apoptosis by diazepam, a non-selective ligand of peripheral benzodiazepine receptors (PBR) which seem to play an important role in apoptosis regulation.

For the study, we used U-87 MG and U373 MG glioma cell lines and primary cultures of GBM cells prepared from peroperatively obtained tumor specimens. The patients included 7 histologically confirmed GBMs. Colorimetric MTT assay was employed to study the photocytotoxic effects of hypericin and diazepam. Flow cytometry was used to detect apoptosis and assess the proapoptotic effects of diazepam.

We found that hypericin upon photoactivation exerts strong cytotoxic effects against U-87 MG and U373 MG cells as well as primary GBM cell cultures. No cytotoxic effect of hypericin was observed under dark conditions. Diazepam inhibited cell growth in U-87 MG cells and primary cultures whereas proliferation of U373 MG cells remained unaffected. When hypericin was combined with diazepam, photocytotoxicity was increased in U-87 MG cells and primary cultures unlike U373 MG cells. Flow cytometric analysis revealed photoactivated hypericin-induced apoptosis in both cell lines. Apoptosis was significantly enhanced by diazepam in U-87 MG cells. However, no such effect was observed in U373 MG cells.

In the present study, we showed that photocytotoxic effect of hypericin in glioma cells can be potentiated by diazepam. This effect is underlied by the ability of diazepam to facilitate hypericin-induced apoptosis. This work provides support to perform clinical studies involving diazepam in the antiglioma treatment regimens as an apoptosis-modulating agent.

Key words: apoptosis, glioma, hypericin, diazepam, peripheral benzodiazepine receptor, photodynamic therapy

Glioblastoma multiforme (GBM) is an extremely deadly neoplasm of the CNS with very unfavourable prognosis resulting in a median survival time of only 40 to 50 weeks [17]. The annual incidence of malignant gliomas (the group consisting of the anaplastic astrocytomas and GBMs) is 3 to 4 per 100 000 population. At least 80 percent of malignant gliomas are GBMs [34]. The triad of currently established treatment options include surgery, radiation therapy and chemotherapy. In spite of encouraging advances in these therapeutical strategies achieved during the last two decades, GBM is still essentially incurable and prognosis of GBM patients remains poor. Therefore, a number of novel treatment modalities are being investigated such as gene therapy, immunotherapy as well as photodynamic therapy (PDT).

The use of PDT to treat gliomas was first investigated by DIAMOND et al in 1972 [12] on glioma cells *in vitro* as well as on animal models. The first attempt at using PDT in treating human gliomas was performed in 1980 by PERRIA et al [32]. In general, antitumoral PDT consists of a systemic administration of a photosensitizer and targeted delivery of visible

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light to the tumor lesion. In the presence of oxygen and upon light stimulation, photosensitizer generates superoxide radicals (type I reaction), which in turn might form peroxide and hydroxyl radicals, or (non-radical) singlet oxygen molecules ($^1O_2$) (type II reaction), events which may result in tumor and/or tumor vasculature destruction [1]. The photosensitizing agents investigated for potential use in brain tumors PDT represent a diverse group of compounds with different chemical structures such as porphyrins, phtalocyanines or polycyclic quinones [33]. One of the most widely studied quinones is hypericin.

Hypericin, a perihydroxylated polycyclic anthraquinone found in plants of Hypericum genus, has been attributed antiretroviral and antitumoral properties when activated by light [1, 27]. The antitumor effects of hypericin are based on its ability to induce apoptosis (and also necrosis) in cancer cells. Hypericin-induced apoptosis was firstly described in cultured human malignant glioma cells [10]. Hypericin-mediated PKC inhibition was suggested to play a key role in inducing apoptosis, however, later it was not confirmed [41]. What has been recognized as one of the first events to occur in hypericin-induced apoptotic cells is mitochondrial damage. Indeed, hypericin has been shown to disrupt mitochondrial transmembrane potential thus inducing permeability transition (PT) [7, 24] as well as to release cytochrome c from mitochodrial intermembrane space into cytosol where it in turn triggers the assembly of a caspase-activating complex termed the apoptosome [39], all being events which irreversibly lead to apoptosis. It was suggested that hypericin induces reactive oxygen species (ROS) generation [37] which preceeds and causes PT resulting in apoptosis [3]. In addition to the intrinsic apoptotic pathway described above also the extrinsic pathway involving cell surface death receptors has been suggested to participate in hypericin-induced apoptosis. Some authors described increased expression of CD95 and CD95 ligand [2, 8] whilst others demostrated an involvement of TRAIL/TRAIL-receptor system [35].

Diazepam is a non-selective ligand of peripheral benzodiazepine receptors (PBR) initially described by BRAESTRUP and SQUIRES [5]. PBR are one of the components of so called "permeability transition pore", a multiprotein complex located on mitochondria and playing a major role in the induction of apoptosis via regulating mitochondrial transmembrane potential [reviewed elsewhere]. Higher PBR density in tumoral versus normal brain tissue has been reported and correlated with cell proliferation, grade of malignancy, and glioma patients prognosis [9, 14, 26]. Some PBR ligands have been shown to facilitate PT/apoptosis induced by anticancer agents [16], TNFα [31], and anti-CD95 antibody [11] or even induce PT/apoptosis on their own [23]. Antiproliferative effects in glioma cells have also been demonstrated [25]. Recently, it was shown that diazepam enhances apoptosis induced by anti-CD95 antibody in Jurkat, however, not in glioblastoma SNB79 cells [11]. In U-87 MG glioma cells, chemotherapy-potentiating and antiproliferative effects of diazepam have been described [22]. For a PBR ligand, PK11195, ability to generate ROS was also demonstrated [13]. Interestingly, PBR seem to be a target of PDT since a close correlation between affinity of porphyrin photosensitizers to PBR and the overall efficacy of PDT was found [40].

Recently, diazepam was investigated in combination with lonidamine (a hexokinase inhibitor and apoptosis inducer) as a novel approach in the treatment of recurrent glioblastoma multiforme [30]. Sixteen patients were included in this phase II trial. Only 7 stabilizations but no complete or partial responses were observed. However, the authors suggest that diazepam might be an interesting agent especially if used in the adjuvant setting or in association with chemotherapy. It appears that a combination of an apoptosis-facilitating agent such as diazepam with an apoptosis-inducing treatment such as chemotherapy or PDT might represent an attractive and potentially effective therapeutical strategy in glioma therapy. Therefore, in this work, we have attempted to enhance hypericin-induced photocytotoxicity and apoptosis by diazepam in glioma cell lines as well as in primary cultures of glioblastoma cells isolated from surgically obtained tumor specimens.

Material and methods

Primary human glioblastoma cell cultures. Seven patients, five males and two females with a mean age of 53 years (range, 28 to 67 years) diagnosed of glioblastoma multiforme at the Department of Neurosurgery, Faculty of Medicine, P.J. Šafárik University, Košice, Slovakia between 1999 and 2000, were included in the study. Histologic diagnosis and classification of glioblastoma multiforme were established using the criteria of the WHO classification [19, 20]. Tumor specimens were obtained peroperatively. The tissues were mechanically disrupted using "Cell Dissociation Sieve – Tissue Grinder Kit" (Sigma, St Louis, MO, USA) and cells were separated by Ficoll-Telebrix density centrifugation (Ficoll Type 400, Sigma; Telebrix^R N 300, Byk Gulden, Konstanz, Germany). Isolated cells were washed twice in DMEM medium (Gibco, Paisley, Scotland, UK) and resuspended in the culture medium: RPMI 1640 with GlutaMAX-I (L-alanyl-L-glutamine, 446 mg.l^{-1}) supplemented with insulin (40 IU.ml^{-1}) (Actrapid^R HM 100 IU/ml, Novo Nordisk, Bagsvaerd, Denmark), apo-transferrin (5 μg.ml^{-1}) (Sigma), 10% foetal calf serum (Gibco), penicillin (100 IU.ml^{-1}) and streptomycin (100 μg.ml^{-1}) (Gibco). Then, viable cells were counted in the Bürker chambers. Cell viability was estimated by trypan blue (Sigma) exclusion. After appropriate adjustment of cell concentration, cells were pipetted into standard 96-well polystyrene cell culture microplates (Sarstedt, Newton, Germany) at c=4–8x10^4 cells per well in a total volume of 60 μl. Cells were maintained at 37 °C in a humidified 5% CO_2/ 95% air incubator (Sanyo, Japan).

Human glioma cell lines. Human glioma cell lines U-87

MG and U373 MG were generously provided by Dr. Christian Gespach (INSERM, Paris, France). Cells were routinely cultured in DMEM containing GlutaMAX-I (862 mg/l) and glucose (4500 mg/l) and supplemented with 10% foetal calf serum (Gibco). Cells were maintained as described above. For experiments, cells were collected from subconfluent monolayers in a solution of trypsin-EDTA (0.5 mg/ml and 0.2 mg/ml, respectively) in PBS without Ca^{2+}, Mg^{2+} (Gibco). Cell viability was estimated by trypan blue exclusion and was greater than 95% before each experiment. Cells were pippeted into 96-well microplates at concentration 10^4 cells/60 µl/well and allowed to attach overnight.

MTT cytotoxicity assay. Cytotoxic effects of hypericin (Molecular Probes, Eugene, OR, USA) and diazepam (Apaurin^R, Krka, Novo mesto, Slovenia) alone or in combination were tested by using the microculture assay with the MTT end-point (MTT=3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) (Sigma). The amount of MTT reduced to formazan is proportional to the number of viable cells [29]. Briefly, concentrations of hypericin and diazepam in the range 10^{-10}–10^{-5} mol.l^{-1} and 10^{-9}–10^{-4} mol.l^{-1}, respectively, were used. Hypericin was prepared by dilution of the stock solution ($c_{hypericin}$=10^{-3} mol.l^{-1} in DMSO; stored at –20 °C) (DMSO purchased from ICN, Aurora, OH, USA) in the culture medium. Diazepam was prepared by dilution of Apaurin^R ($c_{diazepam}$=5 mg.ml^{-1}) in the culture medium. Preliminary experiments were performed to exclude the possibility that solvents (DMSO and ethanol) might influence cell survival. All solutions were prepared freshly before each individual experiment. Then, 40 µl of the tested agents solely or in combinations were added to the cells to achieve desired concentrations in a total volume of 100 µl. Each concentration/combination was added in duplicate. In case of primary glioblastoma cell cultures, one microplate for every patient was kept in darkness and one was irradiated. The irradiation with a total energy density of 100 J.cm^{-2} and power density of 6.9 mW.cm^{-2} was performed using a polychromatic halogen lamp as a source of light. In case of glioma cell lines, experiments under dark conditions were not performed. After 72 hours of incubation, 10 µl of MTT (5 mg/ml) were added in each well. After additional 4 hours, during which insoluble formazan was formed, 100 µl 10% sodium dodecylsulfate (Sigma) were added in each well and another 12 hours allowed the formazan to be dissolved. The absorbance was measured spectrophotometrically at 540 nm using an automated MRX microplate reader (Dynatech Laboratories, Guernsey, UK). Absorbance of the wells containing control (untreated) cells was taken as 100% and cell viability of treated cells was expressed as percentage of the control. For glioma cell lines, six independent experiments were performed.

Detection of apoptosis by flow cytometric analysis. For the assessment of apoptosis induced by hypericin, diazepam or their combinations in U-87 MG and U373 MG cells, we used the Vybrant Apoptosis Assay Kit #1 (Molecular Probes) with a minor modification consisting in replacing annexin V-Alexa 488 with annexin V-FITC. Apoptotic cells are detected on the basis of the externalization of phosphatidylserine which is considered to be a marker of early to intermediate stages of apoptosis. Annexin V binds to the externalized phosphatidylserine whereas SYTOX Green binds to cellular nucleic acids. SYTOX Green is impermeable to live cells and apoptotic cells but stains necrotic cells with intense green fluorescence. Briefly, U-87 MG and U373 MG glioma cells were loaded into the tissue culture Petri dishes (Sarstedt) at density 5x10^5 cells/10 cm^2. The cells were allowed to attach overnight. Then, the culture medium was replaced with the fresh medium containing the indicated concentrations of hypericin, diazepam or their combinations. Subsequently, cells were irradiated as described above. After 72 hours of incubation, both floating and attached cells were harvested, washed twice in PBS, resuspended in the annexin V binding buffer (BD Biosciences, San Diego, CA, USA) and stained with annexin V-FITC (BD Biosciences) and SYTOX Green (Molecular Probes) for 15 minutes in darkness. Afterwards, data acquisition was performed in a FACS Vantage SE flow cytometer (BD Biosciences) using CellQuest Pro software (BD Biosciences), information being stored for 10^4 cells/sample. CellQuest Pro software was also used for data analysis. Cellular debris was excluded from the analysis on the basis of its low right angle light scatter (i.e. side scatter, SSC) properties. Both annexin V-FITC and SYTOX Green fluorescences were detected in the FL1 channel (530/30 band pass filter): viable cells showing little or no (baseline) green fluorescence, apoptotic cells showing green fluorescence (due to annexin V-FITC binding) and late apoptotic/necrotic cells showing a higher level of green fluorescence (due to SYTOX Green binding). For both glioma cell lines, six independent experiments were performed.

Statistical analysis. Statistical analysis was performed using the Arcus QuickStat (Biomedical) software (StatsDirect, CamCode, Sale, UK). To establish the statistical significance of differences observed between various treatments, the one way analysis of variance (ANOVA) employing Tukey-Kramer test for multiple comparisons was used. Statistical significance was considered to be present once p-values were lower than 0.05. The results in figures are expressed as means ±SD from six experiments. Statistical significance is indicated in the figures.

Results

Cytotoxicity of hypericin in primary cultures of glioblastoma cells is light-dependent. Effect of hypericin on viability of primary glioblastoma cell cultures was evaluated under dark and light conditions (Fig. 1). Under dark conditions, hypericin displayed no effect at any of the concentrations tested. We observed only a slight non-significant increase in cell viability at c=10^{-5} mol.l^{-1}. In contrast, after irradiation with 100 J.cm^{-2} of polychromatic light, hypericin signifi-

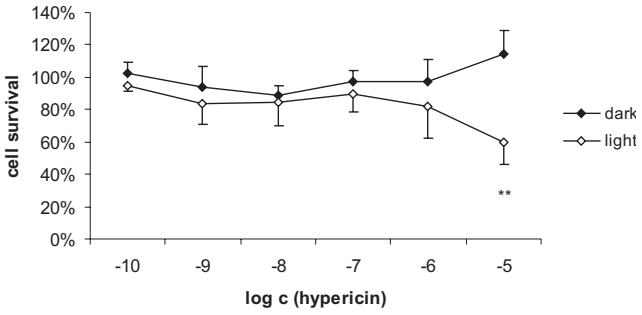

Figure 1. Concentration-dependent effect of hypericin on viability of primary glioblastoma cell cultures under dark versus light conditions. The curves represent mean values ±SD. **p<0.01 dark vs. light.

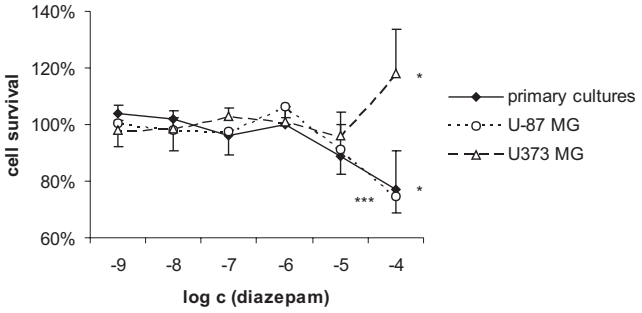

Figure 2. Concentration-dependent effect of diazepam on viability of primary glioblastoma cell cultures, U-87 MG and U373 MG glioma cell lines. The curves represent mean values ±SD. *p<0.05 ***p<0.001 diazepam at c=10^{-4} mol.l^{-1} vs. no treatment.

cantly affected cell viability reducing cell survival by around 40% (p<0.01), however, only at the highest concentration (10^{-5} mol.l^{-1}) used. For this reason, we selected only the two highest concentrations of hypericin, namely 10^{-6} and 10^{-5} mol.l^{-1}, to be tested in glioma cell lines as well as in combined experiments.

Diazepam affects the growth of human glioma cells. Concentrations of diazepam ranging from 10^{-9} to 10^{-4} mol.l^{-1} were tested for their antiproliferative effects on primary glioblastoma cell cultures, U-87 MG and U373 MG glioma cell lines (Fig. 2). Diazepam at 10^{-9} to 10^{-5} mol.l^{-1} proved to be ineffective in terms of influencing the growth of primary cultures as well as both cell lines. However, after incubation with diazepam at 10^{-4} mol.l^{-1}, the growth of primary glioblastoma cell cultures and U-87 MG cells was significantly inhibited by 23% (p<0.05) and 25% (p<0.01), respectively. In contrast, the same concentration of diazepam caused a significant 18% increase (p<0.05) in the growth of U373 MG cells as assessed by the MTT test. With respect to these results as well as our previous work [22], we selected the two highest concentrations of diazepam for further experiments.

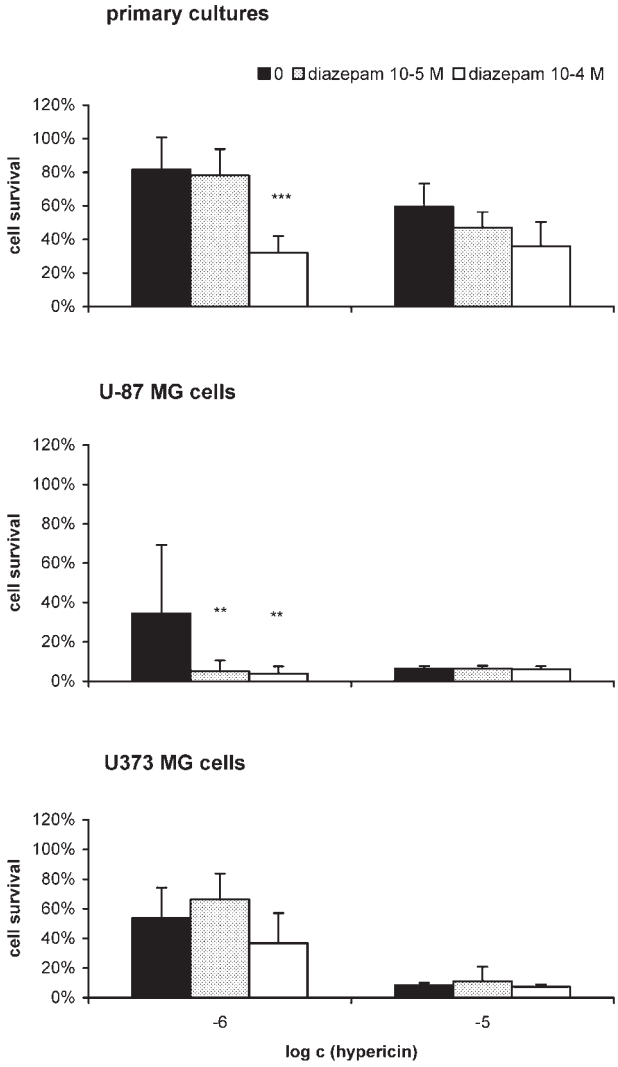

Figure 3. Phototocytotoxic effect of combinations of hypericin and diazepam in primary glioblastoma cell cultures, U-87 MG and U373 MG glioma cell lines. Addition of diazepam to hypericin potentiates the photocytotoxic effect of the latter in primary cultures as well as U-87 MG cells. The bars represent mean values ±SD. **p<0.01 ***p<0.001 hypericin alone vs. combined treatments.

Diazepam potentiates hypericin photocytotoxicity. For the assessment of diazepam capacity to enhance the photocytotoxicity of hypericin, we combined both agents using the concentrations 10^{-5}–10^{-4} and 10^{-6}–10^{-5} mol.l^{-1} of diazepam and hypericin, respectively (Fig. 3). We observed different sensitivity to hypericin photocytotoxic effects in different glioma cell types. The sensitivity increased in the following order: primary cultures of glioblastoma cells <U373 MG cells <U-87 MG cells, with U-87 MG cells being the most sensitive. In primary glioblastoma cell cultures and U-87 MG cells, a significant (p<0.001 and p<0.01, respectively) potentiation of hypericin photocytotoxicity was observed when di-

azepam at 10^{-4} mol.l^{-1} was added to hypericin at 10^{-6} mol.l^{-1}. The effect of the combination of both agents was significantly higher than the effects of each agent alone. Similar effect was observed if lower concentration of diazepam, 10^{-5} mol.l^{-1}, was combined with hypericin at 10^{-6} mol.l^{-1}, however only in U-87 MG cells. On the contrary, photocytotoxic effect of higher hypericin concentration, 10^{-5} mol.l^{-1}, was not significantly influenced by the addition of diazepam in any of the cell types used though there was a tendency (non-significant) to such an effect in primary glioblastoma cell cultures. This might be due mainly to a very high photocytotoxic activity of 10^{-5} mol.l^{-1} hypericin which alone causes 94% and 91% reduction in survival of U-87 MG and U373 MG cells, respectively. In contrast to U-87 MG cells and primary glioblastoma cell cultures, no potentiation of hypericin photocytotoxicity by diazepam was observed in U373 MG cells as assessed by the MTT test. On the basis of these results, we have chosen 10^{-6} mol.l^{-1} hypericin and 10^{-4} mol.l^{-1} diazepam to be tested in the subsequent apoptosis experiment.

Diazepam enhances apoptosis induced by photoactivated hypericin. To test the hypothesis that potentiation of hypericin photocytotoxicity by diazepam can be attributed to diazepam's ability to enhance hypericin-induced apoptosis, we used a flow cytometric analysis and evaluated the extent of apoptosis and necrosis by annexin V and SYTOX Green staining, respectively (Fig. 4 and 5). Apoptotic cells were identified on the basis of their medium-intense green fluorescence (due to annexin V-FITC binding) whereas late apoptotic/necrotic cells were recognized as those events displaying high-intensity green fluorescence (due to SYTOX Green binding) as demonstrated in Figure 4. In both U-87 MG and U373 MG cells, hypericin at 10^{-6} mol.l^{-1} alone or in combination with 10^{-4} mol.l^{-1} diazepam induced a significant reduction in the number of viable cells which was accompanied by a significant increase in the amount of apoptotic as well as late apoptotic/necrotic cells (Fig. 5). In contrast, neither apoptosis nor necrosis was detected when cells were treated with diazepam alone regardless of its concentration (not shown). In U-87 MG cells, the combination of hypericin and diazepam proved to be significantly ($p<0.05$) more effective in inducing apoptosis than hypericin alone (Fig. 4D, Fig. 5). Interestingly, only apoptosis unlike necrosis was facilitated by diazepam in these cells. On the other hand, no facilitation of either apoptosis or necrosis occurred in U373 MG cells.

Discussion

In the present work, we have shown that hypericin after photoactivation possesses strong photocytotoxic activity and induces apoptosis in human glioma cell lines as well as glioma cells isolated from surgical human tumor specimens in concentration-dependent manner which is in agreement with other authors' results. Moreover, we have clearly demonstrated that these effects of hypericin are enhanced by diazepam. We also demonstrated that the underlying mechanism of hypericin photocytotoxicity potentiation is the enhancement of apoptosis. Thus, diazepam can be assigned to possess apoptosis-facilitating properties, at least in glioma cells. Until now, so eminent a quality has been reserved only for other PBR ligands such as PK11195, Ro5-4864 or FGIN-1-27.

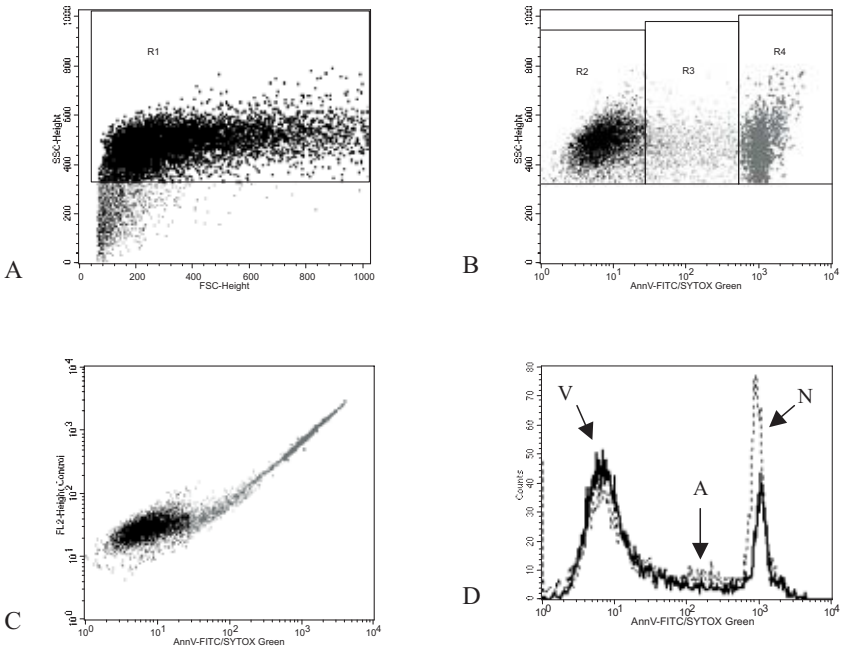

Figure 4. Illustrative bivariate dot plots and a histogram of the flow cytometric analysis of apoptosis induced by photoactivated hypericin (c=10^{-6} mol.l^{-1}) in U-87 MG glioma cell line as determined by annexin V-FITC and SYTOX Green staining. A, dot plot showing all events: region R1 represents whole cells (highlighted black dots) while cellular debris displaying lower SSC was excluded from analysis; B, dot plot showing whole cells only (R1-gated): regions R2, R3 and R4 represent viable cells (black dots), apoptotic cells (grey dots) and late apoptotic/necrotic cells (highlighted grey dots), respectively; C, whole cells (R1-gated) displayed on a FL1-FL2 bivariate dot plot (non-compensated): different types of dots represent the same regions as in the dot plot B; D, histogram comparing effects of hypericin alone (full line) and hypericin in combination with diazepam (c=10^{-4} mol.l^{-1}) (dashed line). Peaks representing viable, apoptotic and necrotic cells are indicated by letters V, A and N, respectively. The peaks clearly show a decline in viable cell population with a concomitant increase in apoptotic as well as necrotic cell populations when hypericin is combined with diazepam.

Several mechanisms underlying this potentiation might be proposed. Firstly, it was shown that hypericin induces PT [7, 24]. For diazepam, PT facilitating effects have been demonstrated [11]. Furthermore, diazepam could possibly contribute to hypericin-induced ROS production though amongst PBR ligands, oxidative stress has only been associated with PK11195 [13]. Diazepam was also shown to enhance cytotoxicity of etoposide [22], lonidamine [25], and anti-CD95 antibody [11]. Interestingly, hypericin is able to increase expression of both CD95 and CD95 ligand [2, 8]. In contrast, SCHEMPP et al [35] showed that hypericin photo-induced apoptosis are at least in part mediated by TRAIL/TRAIL-receptor system rather than FasL/Fas (i.e. CD95L/CD95) system. Finally, a close correlation between PDT efficacy and photosensitizers' affinity to PBR was revealed what might indicate an involvement of PBR in PDT mechanisms [18, 40]. PK 11195 was also shown to block P-glycoprotein-mediated efflux in acute myeloid leukemia cells [4] and to increase mitochondrial uptake of a photosensitizer in FaDu cells [28]. However, hypericin is neither a Pgp substrate nor it seems to be accumulated in mitochondria of glioblastoma cells [38]. Also, no interaction has been described between Pgp and diazepam. Therefore we do not consider this mechanism to be likely involved in diazepam-mediated potentiation of hypericin-induced apoptosis. Based on the mitochondrial localization of PBR receptors and predominantly mitochondrial mechanism of hypericin action, we suppose that the proapoptotic properties of diazepam might stem most likely from the facilitation of PT induction though it was not directly demonstrated in the present work.

Interestingly, we found differential effects of diazepam in U-87 MG and U373 MG cell lines while the effects of hypericin were comparable. Interestingly, in U-87 MG cells diazepam inhibited cell proliferation, potentiated photocytotoxic effect of hypericin and facilitated hypericin-induced apoptosis. On the other hand, in U373 MG cells diazepam promoted rather than inhibited proliferation which was reflected in its incompetence to enhance both phototoxicity and apoptosis induced by hypericin. It is known that both cell lines have similar chemosensitivity but U-87 MG cells express the wild-type p53 whereas U373 MG cells express p53 of the mutant type. However, it was demonstrated that p53 plays no role in the photocytotoxicity of hypericin [41]. Our findings are consistent with these observations. We have also tested the influence of pifithrin-alpha, a p53 inhibitor [21], on the effects of hypericin and diazepam but no effect was observed (unpublished data). U-87 MG and U373 MG cells also differ in the expression of PBR. CARAYON et al [6] detected by flow cytometry $4.96\pm0.24 \times 10^5$ PBR sites per cell in U-87 MG but only $3.21\pm0.51 \times 10^5$ PBR sites per cell in U373 MG cells. We assume that different PBR densities rather than p53 might be responsible for diverse effects of diazepam in the two glioma cell lines. If diazepam is to be used as a part of multimodal antiglioma therapies, it might be useful to know prospectively the level of PBR expression in a particular patient.

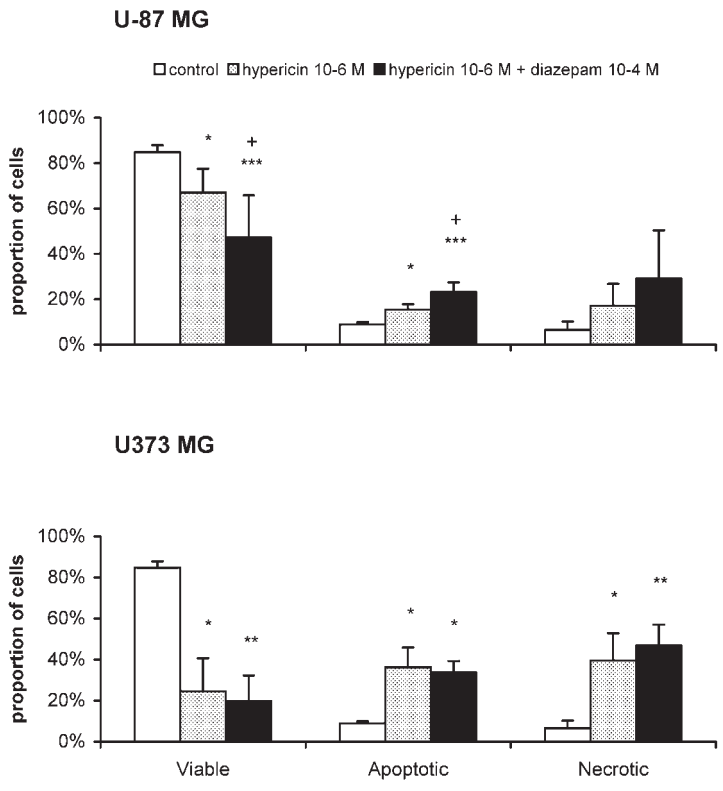

Figure 5. Results of flow cytometric analysis of the effect of diazepam ($c=10^{-4}$ M) on apoptosis induced by photoactivated hypericin ($c=10^{-6}$ M) in U-87 MG and U373 MG glioma cell lines. The bars represent mean values ±SD. V, viable cells; A, apoptotic cells; N, late apoptotic/necrotic cells. $^{*}p<0.05$ $^{**}p<0.01$ $^{***}p<0.001$ treatments vs. control, $^{+}p<0.05$ combined treatments vs. hypericin alone.

In clinical practice, hypericin and diazepam have been used to treat various CNS disorders such as depression, anxiety, epilepsy etc. Either drug should be able to accumulate in the brain tissue in sufficient concentrations shown to possess effects against glioma though direct evidence for such accumulation has not been provided yet [15, 36]. The first clinical trial with hypericin in the treatment of malignant gliomas was completed in 1998 but to the best of our knowledge the results have not been published. A phase II study was recently performed combining lonidamine and diazepam in the treatment of recurrent glioblastoma multiforme [30]. The authors observed no complete or partial responses, however 7 of 16 patients demonstrated stabilization of the disease.

We suggest that hypericin and diazepam have cer-

tain potential to be exploited in the treatment of human gliomas in the future. We assume that diazepam might be particularly useful in combination with chemotherapy as an antineoplastic drug-induced apoptosis facilitating agent.

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