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Modulatory Effects of Curcumin and Tyrphostins (AG494 and AG1478) on Growth Regulation and Viability of LN229 Human Brain Cancer Cells

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In this study we employed curcumin as a potent adjuvant agent in the treatment of human brain cancer involving selective EGFR kinase inhibitors: tyrphostins AG494 and AG1478. Aim of this work was to evaluate the effect of tested compounds on autocrine growth, cell cycle, and viability of LN229 cells, as well as to assess their proapoptotic and genotoxic properties. Our results showed that all tested compounds significantly inhibited autocrine growth of the investigated cell line in a dose dependent manner. However they are characterized by different kinetics of cell growth inhibition. Suppression of growth by the tyrphostins was completely or partially reversible in contrast to curcumin. Curcumin increased the cytostatic and/or cytotoxic potential of AG494 and AG1478. Tyrphostins did not have genotoxic properties regardless of concentration used, whereas curcumin cytotoxic and genotoxic properties were directly proportional to the concentration. Curcumin significantly increased tyrphostins cytotoxicity. The most promising of the obtained results may be the use of curcumin and tyrphostin AG494 in the treatment of cancer cells. Anticancer effect of the mixture was confirmed by increase of cytotoxic effect, decrease of viability, stimulation of apoptotic procesess, irreversible DNA damage, and decrease of the ROS in the culture of glioblastoma cells.

INTRODUCTION

Human brain cancer (*Glioblastoma multiforme*; GBM) is a relatively rare cancer. The incidence rate for is approximately 3–4/100,000 person-years. Rates generally tend to be higher in developed, industrial countries (1). However GBM is characterized by infiltrative growth, intensive migration, and the rapid progression of the cancer cells within the surrounding nerve tissue. That kind of tumor's growth impedes the total surgical resection of glioblastoma, hence recurrence of tumors is commonly observed (2,3). The 5-yr survival after standard therapy (chirurgical resection of the tumor, radiation, chemotherapy with temozomolide) is rare (2–5%) and life expectancy after diagnosis is poor (6 months to 1,5 year) (2,4). Therefore it is crucial to improve already existing therapies and to find alternative methods of treatment.

Protein tyrosine kinases (including growth factor receptors tyrosine kinases) play a crucial role in the regulation of many cellular processes such as growth, differentiation, and die of normal and cancer cells. An increased production of transforming growth factor α (TGFα) or epidermal growth factor (EGF) and its receptor (EGFR) was found in several cancer cell lines (5,6), including human brain cancer (LN229) (7). One of the ways of inhibition the transduction signal via EGFR (the so-called *autocrine loop*) are tyrosine phosphorylation inhibitors (tyrphostins) (8,9). Constitutive oncogenic activation in cancer cells can be blocked by selective (e.g., tyrphostins) or nonselective (e.g., polyphenols) inhibitors. Supplied with daily diet, polyphenol curcumin (spice, food coloring E100) is characterized by anticancer and cytoprotective properties, due to (as well as tyrphostins) the ability to interact with EGFR (10). Thus it was decided to evaluate the effectiveness of potential targeted therapy of EGFR's inhibitors and curcumin.

In the present work, we tested 2 tyrphostins: AG494 and AG1478 in the autocrine growth regulation of human brain cancer cells (*Glioblastoma multiforme*; LN229; derived from cancerous brain cortex tissue). Both the investigated compounds are specific ATP-competitive inhibitors of tyrosine kinase, located in the intracellular domain of the EGFR. However, tyrphostin AG1478, unlike AG494, acts in a nonreversible manner (8,9,11). Also, AG1478 structurally is very similar to clinically used erlotinib (TarcevaTM) and gefitinib (IressaTM), which implies it has a potent anticancer

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application. We tried to evaluate the tyrphostins and curcumin influence on proliferation, viability, and genotoxicity of LN229 cells.

MATERIALS AND METHODS

Chemicals and Reagents

Dulbecco's modified minimal essential medium (DMEM), fetal bovine serum (FBS), 10,000 IU/ml penicillin, and 10 mg/ml streptomycin were purchased from PAA Laboratories GmbH (Pasching, Austria). Bovine serum albumin, transferrin, trypsin, taxol, Hoechst No 33342, Crystal Violet, propidium iodide, and tyrphostins: AG494 and AG1478 (kinase inhibition IC_{50}: 1.2 μM and 3 nM, respectively, values were taken from the manufacturer's leaflets), dimethyl sulfoxide (DMSO), ethylenediaminetetraacetic acid, [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MTT] were obtained from Sigma (St. Louis, MO). Curcumin (C; Diferuloylmethane; Fluka, Switzerland); other reagents were provided by POCh (Gliwice, Poland). Stock solutions of the tyrphostins were prepared by dissolving the compounds in DMSO to a final concentration of 10 mM (AG1478) and 20 mM (AG494), whereas curcumin was dissolved in ethanol to a final concentration of 10 mM. The resultant solutions were stored at −20°C. DMEM/F12 (1:1) medium was used to dilute stock solutions to working concentrations (AG494: 1–20 μM, AG1478: 0.1–8 μM, curcumin 5–12 μM).

Cell Culture

LN229 (CRL-2611) cells were obtained from the American Type Culture Collection (Virginia, USA) and cultured in DMEM in the presence of 10% FBS. The cells were passaged two or three times per week.

Cell Proliferation Assays

Target cells were seeded on 96-well plates at concentrations of 5×10^3 cells/well in DMEM, supplemented with 100 IU/ml penicillin and 100 μg/ml streptomycin in the presence of 10% FBS. Following 24 h of incubation, the culture medium was replaced with serum-free DMEM/F12 (1:1) supplemented with transferrin (5 mg/ml), sodium selenite (2 ng/ml), and albumin (0.5 mg/ml; DMEM/F12+). After an additional 24 h of incubation (Day 0), the medium was replaced by serum-free DMEM/F12+ medium containing tyrosine kinase inhibitors: AG494, AG1478 and curcumin respectively in concentration ranges 1–20 μM, 0.1–8 μM, and 5–12 μM. The incubation was continued for the 48 h at 37°C in a humidified atmosphere. The modified crystal violet staining method (CV) (12) and MTT assay (13) were used to determine the influence of the tyrphostins and curcumin on the proliferation of target cells. The absorbance at a wavelength of 570 nm or 540 nm (MTT and CV assay respectively) was measured using a Tecan (Spectra Fluor Plus) multiscan plate recorder. Five replicate wells were used for each of the 3 independent experiments for each method. The effect of tyrosine kinase inhibitors and curcumin were expressed as a relative (to the controls) decrease in cell growth determined after 48 h of incubation with the investigated compounds. The calculated parameter was:

$$G_i = \frac{\overline{A_i} - \overline{A_0}}{\overline{A_k} - \overline{A_0}} \times 100\%$$

where: Gi = growth inhibition, A = average values of absorbance: control at the start of experiments ($A0$), and after the time of incubation without (Ak) and with (Ai) the investigated compounds.

Growth Resumption After Treatment with Tyrphostins and Curcumin

Initial conditions (cell number, growth medium, type of plates, and time of incubation without inhibitors) were the same as described above (see Cell Proliferation Assays). After determining the number of cells in Day 0, the rest of the cells cultures were treated with tyrphostins AG494 and AG1478 and curcumin in concentration previously outlined as IC_{50} (12.34; 0.17 and 7.1, μM respectively) and IC_{90} (20.3; 1.6 and 10.13, μM respectively). At the same time, the control cells were prepared, by culturing them in analogous conditions in DMEM/F12+. After 24 h of incubation in serum-free medium with inhibitors, the half of the plate was replaced with fresh DMEM/F12+ without tyrphostins and curcumin, and the second half, with the investigated compounds. After additional 48 and 72 h of incubation (78 and 102 h from the Day 0) the number of cells was determine by the MTT method. Six replicate wells (per concentration) were used for each of three independent assays.

Evaluation of Cytostatic Properties of Curcumin and Tyrphostins Mixtures

The MTT test was used to determine whether curcumin modulates the antiproliferative properties of AG494 and AG1478 tyrphostins. Initial conditions (cell number, growth medium, type of plates, and time of incubation without inhibitors) were the same as described in Cell Proliferation Assays. After determining the number of cells in Day 0, the rest of cells were treated with tyrphostin AG494 or AG1478 in concentration IC_{50} (12.34 and 0.17 μM, respectively) and then curcumin in concentrations 2, 4, 6, 8, and 10 μM. The mean absorbance value for each inhibitor concentration was estimated. Three independent experiments were done, each performed in 2 replicates.

Cell Cycle Analysis

To estimate the proportion of cells in various phases of cell cycle, cellular DNA contents were measured by flow cytometry (FACS). LN229 cells (2.5×10^5/dish) were plated on a Petri dish (Ø 6 cm) in 5.0 ml DMEM supplemented with 10% FBS. The next day, the culture medium was replaced with serum-free DMEM/F12+ medium. After an additional 24 h of incubation, the cells were exposed to the investigated compounds and their mixtures in concentrations outlined previously as IC_{50}. The experiment was carried out for 48 h and then cells were harvested by trypsinization and centrifuged. The preparation of the cells to analyze their cell cycle was performed according to the protocol described previously (14). Three independent experiments were performed. DNA content was determined using a FACScan Beckton-Dickinson flow cytometer and obtained data was analyzed by FCS Express 5 Plus Research Edition program.

Assessment of Cell Viability

The double staining method (Hoechst 33342/PI) (11) was used in the investigation of the effect of tyrphostins and curcumin on the viability of LN229 cells. The cells were seeded on 24-well plates at a density of 2.5×10^4/well in 1.0 ml DMEM with 10% FBS. The next day, the culture medium was replaced with serum-free DMEM/F12+ medium. After an additional 24 h of incubation, the cells were exposed to the investigated compounds in concentrations outlined previously as IC_{50} and $2 \times IC_{50}$. As a negative control served cells cultured in analogous conditions in DMEM/F12+, whereas the positive control cells were cultured with paclitaxel (PTX) in concentration 5 and 10 nM (IC_{50} and $2xIC_{50}$, respectively). Cells were also treated with the following mixtures: AG494+AG1478, C+AG494 and C+AG1478. The inhibitors were diluted in such a way to obtain mixtures, in which each of the compounds was in concentration of IC_{50} (A) or $2xIC_{50}$ (B). The experiment was carried out for 48 h and then Hoechst 33342 and propidium iodide were added. The double staining method was performed according to the protocol described previously (14). Four replicate wells (per concentration) were used for each experiment. Three independent experiments were performed.

Determination of Effector Caspases 3/7 Activity

To determine the activity of caspases 3/7 after treatment with curcumin, tyrphostins and their mixtures the Promega Caspase-Glo® 3/7 kit was used. Initial conditions (cell number, growth medium, and time of incubation without inhibitors) were the same as described above (see Cell Proliferation Assays). However cells were seeded on 96-well sterile white plates. The cells were exposed to the investigated compounds in concentrations $2 \times IC_{50}$. As a negative control served cells cultured in analogous conditions in DMEM/F12+ with 1 mM DMSO (solvent of tested compounds), whereas the positive control cells were cultured with PTX in concentration 10 nM ($2 \times IC_{50}$). Cells were also treated with the following mixtures: AG494+AG1478, C+AG494, and C+AG1478. The inhibitors were diluted in such a way to obtain mixtures, in which each of the compounds was in concentration of IC_{50}. After 12 and 24 h the cells were lysed with PBS and lysis buffer (10:1; 25 μl/well). Then we continued the experiment according to the protocol (15), which was provided by the kit manufacturer. The luminescence was measured using a Tecan (Spectra Fluor Plus) multiscan plate recorder at gain 120 and integration time of 1000 ms. The mean luminescence value for each inhibitor concentration was estimated. Three independent experiments were done, each performed in 2 replicates.

ROS Assay

The assay with 2′,7′-Dichlorofluorescin diacetate (DCFH2-DA) (16) was performed to indicate the ability of tested compounds to induce or scavenge the reactive oxygen species (ROS) within cells. Initial conditions (cell number, growth medium, type plates, and time of incubation without inhibitors) were the same as described above (see Cell Proliferation Assays). LN229 cells were exposed to the investigated compounds in concentrations outlined previously as IC_{50} and $2 \times IC_{50}$. As a negative control served cells cultured in analogous conditions in DMEM/F12+. Cells were also treated with the following mixtures: AG494+AG1478, C+AG494, and C+AG1478. The inhibitors were diluted in such a way to obtain mixtures, in which each of the compounds was in concentration of IC_{50} (A) or $2 \times IC_{50}$ (B). After a 30-min incubation the 10 μl/well of hydrogen peroxide (H_2O_2) in 20 μM concentration was added. The incubation was continued for the next 30 min at 37°C in a humid atmosphere and then 10 μl/well of DCFH2-DA was added. Then plates were incubated 10 min in darkness in 37°C. After that medium was replaced with PBS (100 μl/well).

The emission of fluorescence at $\lambda = 535$ nm with excitation of $\lambda = 470$ nm wavelength was measured using a Tecan (Spectra Fluor Plus) multiscan plate recorder. The mean fluorescence value for each inhibitor concentration was estimated. Three replicate wells (per concentration) were used for each experiment. Five independent experiments were performed.

Genotoxicity Assay

The Single Cell Gel Electrophoresis (SCGE; Comet Assay) was used to indicate whether the tyrphostins and curcumin were able to induce DNA damage in LN229 cells. If so, whether this process is reversible or not. The experimental conditions (cell number, growth medium, type of plates, time of incubation with inhibitors) were the same as described above (Assessment of Cell Viability). In addition, the cells intended to be tested for reversibility, after 48 h of incubation

with inhibitors and their mixtures, were rinsed with a fresh growth medium. Then cultures were left for 1 or 4 h in a defined medium without inhibitors. Afterwards cells were trypsynized and comet assay was performed, according to the protocol described in (17). Two replicate wells (per concentration) were used for each experiment. Three independent experiments were performed. Results were shown as the mean DNA damage (%), which is a percentage of the DNA that migrated to the comet's tail.

Statistical Analysis

Statistical analysis was performed with the use of the STATISTICA 10 program. The effects of inhibitors concentrations on LN229 cells proliferation were analyzed using 1-way analysis of variance, followed by Dunnett's test. The Mann–Whitney U test was used to determine differences in absorbance of samples incubated with given inhibitor versus samples in which the inhibitor was removed. Differences in the percentage of cells in given phase of cell cycle were analyzed using 1-way ANOVA followed by Fishers least significant difference test (LSD). To determine differences in the percentage of alive and apoptotic cells in the control sample versus tyrphostins, PTX, and their mixtures treated samples the ANOVA, followed by Tukey's honest significance difference test were used. Statistical significance between percentage of DNA damage in the control samples and tyrphostins and their mixtures treated samples was calculated by ANOVA, followed by Tukey's honestly significant difference (HSD) test.

RESULTS

Effect of Tyrphostins (AG494, AG1478) and Curcumin on LN229 Proliferation

Human brain cancer cells LN229 were exposed for 48 h to tyrosine kinase inhibitors, added at the concentration range of 1–20 μM (AG494), 0.1–8 μM (AG1478), and 5–12 μM (curcumin). Three repetitions were performed for each method. The effect of the investigated tyrphostins and curcumin determined by CV and MTT assay is shown in Fig. 1. The exposure of LN229 cells to tyrphostins and curcumin resulted in a significant dose-dependent suppression of proliferation compared to the control cultures. Autocrine growth of the investigated cells, determined by the CV and MTT method, was practically completely inhibited by AG1478 at concentration ≥ 2 μM and by curcumin at an concentration ≥ 12 μM.

While tyrphostin AG494 was unable to complete inhibit the growth of *Glioblastoma* cells, regardless of concentration used and the number of cells decreased to about 90% of control. This might confirm that AG494 act in reversible manner. Inhibition curves of AG494 and curcumin were similar, what might indicate the influence on the same (or similar) mitogenic signaling pathway. Tyrphostin AG1478 unlike to other 2

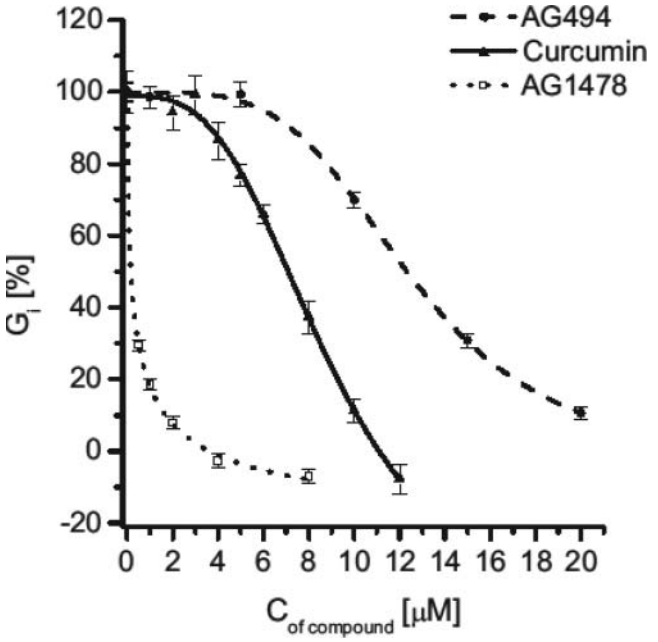

FIG. 1. The dose-dependent effect of tyrphostins: AG494, AG1478, and curcumin on the autocrine growth of LN229 cells after 48 h of incubation in serum-free DMEM/F12+ medium. Each point is the average of the values determined by MTT and CV assay. G_i = growth inhibition; C = concentration of the investigated compound.

compounds probably induce a different mitogenic signals. Comparison of the growth inhibition effect of the investigated compounds was shown in Table 1.

A 10%, 50%, and 90% inhibition of the growth of LN229 cells was determined by fitting a sigmoidal model of the dose-dependent effect of the investigated inhibitor. IC_{10}, IC_{50}, and IC_{90} coefficients calculated from the growth inhibition curves. Tyrphostin AG1478 was a more potent inhibitor of *Glioblastoma* cells growth than AG494 (100×) and curcumin (40×).

Growth inhibition effect of the investigated tyrphostins was partially (AG494) or completely (AG1478) reversible (Fig. 2). The human brain cancer cells, inhibited by tyrphostin AG494, were able to resume proliferation. AG494 in IC_{50} concentration (12.34 μM) did not cause the nonreversible inhibition of growth (there were no statistically significant differences in relative to proliferation of control cells). However, after exchange

TABLE 1
Values of IC_{10}, IC_{50}, and IC_{90} calculated from inhibitor curves of AG494, AG1478 and curcumin

	IC coefficient [μM]		
Inhibitor	IC10	IC50	IC90
AG494	7.18 ± 2.98	12.34 ± 0.82	20.29 ± 1.65
AG1478	0.01 ± 0.82	0.17 ± 0.13	1.64 ± 1.09
Curcumin	3.67 ± 2.55	7.1 ± 1.26	10.13 ± 1.33

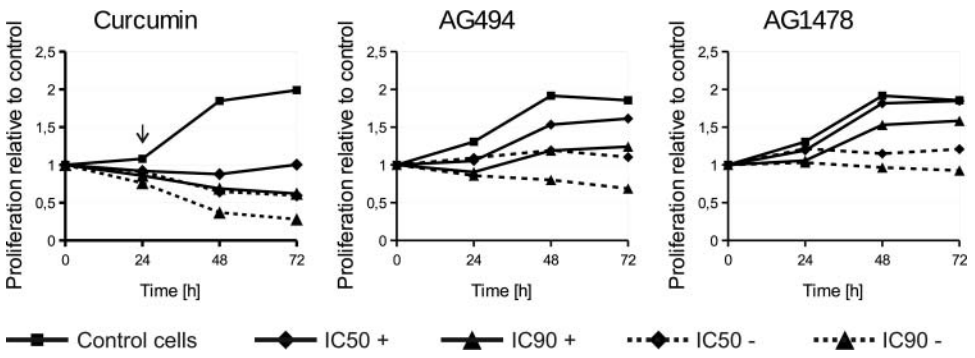

FIG. 2. Reinitiation of LN229 cells autocrine growth, after replacement of the AG494 (A), AG1478 (B), and curcumin (C) with fresh culture medium. I- cells = cells in which media with inhibitor were replaced with fresh growth media;, I+ cells = cells that were incubated with inhibitor by the whole time of the incubation. Each inhibitor was added at concentrations IC_{50} and IC_{90}. Each point is an average of three independent experiments. Vertical bars = 95% confidence interval with Bonferroni correction.

for fresh DMEM/F12+ medium, the rate of cells proliferation (the slope of the curve) was significantly lower than in control cells. In contrary, growth arrest caused by tyrphostin AG1478 was completely reversible. The proliferation rate of cells inhibited by AG1478 (after exchange with the fresh defined medium) returned to the control values. Out of the tested compounds, only curcumin in concentration of IC_{90} caused completely inhibition of *Glioblastoma* cells growth. However, it is seen that number of cells is slightly increasing after inhibitor's removal from the medium, but it is significantly lower than in control cells. Moreover, in control cultures we observed 2× or 4× increase of cells number than in cultures with curcumin IC_{50} I- as well as in IC_{50} I+. This allowed to draw a hypothesis, that curcumin overall changes kinetics of LN229 cell proliferation. It is possible that investigated polyphenol in these concentration is cytotoxic to human brain cancer cells.

Influence of Curcumin on Inhibition of LN229 Cell Proliferation by Tyrphostins AG494 and AG1478

The MTT test was also used to evaluate how curcumin modulate antiproliferative properties of the investigated tyrphostins. Results are shown in Fig. 3. Considering the modulating effect of curcumin on LN229 cells treated with AG494 after 48 h of incubation in serum free medium, it can be concluded that curcumin in concentration range 4–12 μM modulate the growth inhibition properties of the tyrphostin. The shape and the slope of the inhibition curve were similar to the one characteristic to the curcumin alone. However curcumin used in concentration $\geq$8 μM decreased growth inhibition below the D0 value. It indicates that AG494–curcumin mixture was cytotoxic to human brain cancer cells, in contrast to AG494 and curcumin alone (Fig. 1). Curcumin in concentration 2–6 μM had no effect on growth inhibition of *Glioblastoma* cells caused by tyrphostin AG1478. It may be assumed that in that concentration range polyphenol act like protective agent on investigated cells. Although curcumin alone in concentration 2–6 μM caused 20% decrease of proliferation compared to the control (Fig. 1). On the other hand, curcumin in concentration $\geq$8 μM increased the growth inhibition of LN229 cells caused by AG1478 used in concentration IC_{50} (Fig. 1). In case of modulatory effect of curcumin on growth inhibition caused by AG1478, it is clearly seen that tyrphostin is responsible for growth arrest, even at its lowest concentration used.

Effect of Tested Compounds and Their Mixtures on Cell Cycle of LN229

DNA content of *Glioblastoma* cells was determined by the use of a flow cytometer FACScan Beckton-Dickinson after 48 h of incubation with the investigated compounds in

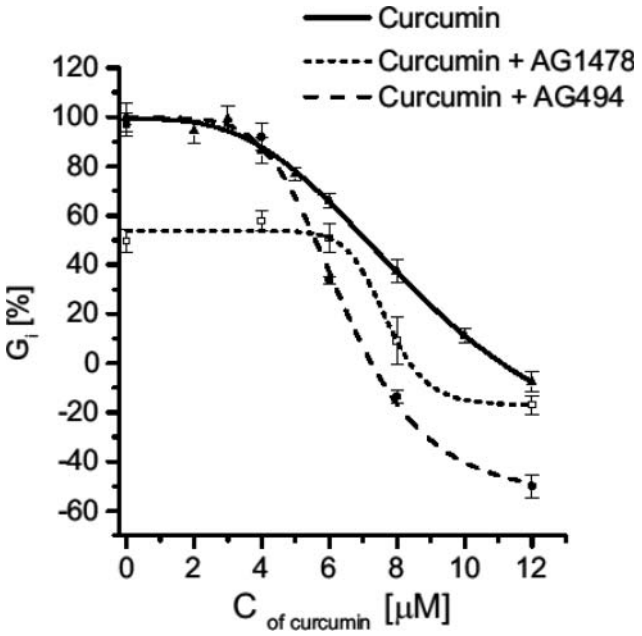

FIG. 3. Influence of curcumin on tyrphostins AG1478 and AG494 (both in IC_{50} concentration) induced growth inhibition of LN229 cells after 48 h incubation in serum free MDEM/F12+. Each point is an average of three independent experiments. Vertical bars, SD.

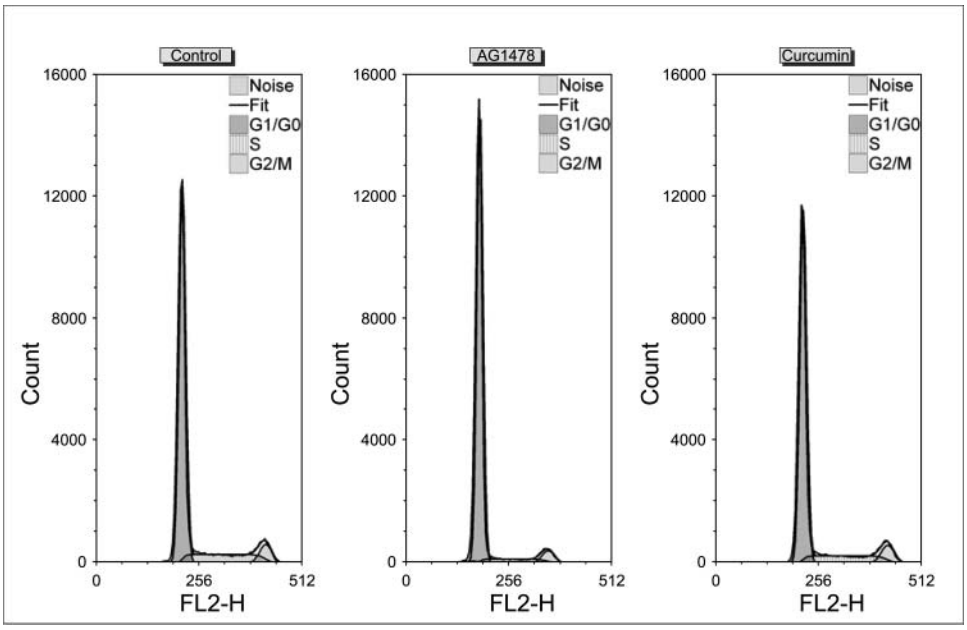

FIG. 4. Influence of tyrphostin AG1478 and curcumin on DNA content in the various phases of cell cycle of LN229 cells. Cellular DNA contents were measured by flow cytometry after 48 h of incubation with compounds in IC_{50} concentration, dissolved in serum free DMEM/F12+ medium.

concentration IC_{50}. The typical distribution of the cells in various phases of cell cycle is shown in Fig. 4, whereas quantitative results (percentage of the cells in the given phase of cell cycle) are shown in Fig. 5. Tyrphostin AG494 and curcumin do not affect the cell cycle of LN229, unlike the tyrphostin AG1478. That EGFR inhibitor in statistically significant manner decreased the cell number in G2 phase by 7% and increased the number of cells in G1 phase by 15% (in relation to control). Thus *Glioblastoma* cells were arrested in G1/G0 phase. Similar effect was observed in the cells treated with mixture of both tyrphostins and in mixture of AG1478 with polyphenol. The increase of cell number in G1 phase was 17% and 12%, respectively, therefore cells were also arrested in cell cycle in G1 phase. Probably the observed phenomenon was caused by AG1478. In case of mixture of AG494 with curcumin the effect is quite opposite. The number of cells in G1 phase was decreased by 20% in relation to control, whereas the number of cells in S phase was increased by 19% in relation to control. Presented data indicate that cytostatic properties of AG1478 were underlying in cell cycle arrest in G1/G0 phase. Whereas mechanism of growth inhibition caused by AG494 and curcumin was different than cell cycle disruption.

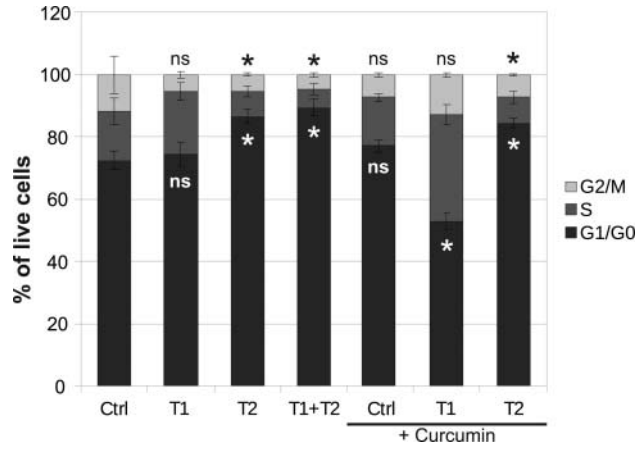

FIG. 5. The effect of tested compounds and their mixtures on the proportion of *Glioblastoma* cell line in various phases of cell cycle. Cellular DNA contents were measured by flow cytometry after 48 h of incubation with compounds in IC_{50} concentration, dissolved in serum-free DMEM/F12+ medium. Each bar is an average of 3 independent experiments. Vertical bars, SD. Ctr = control cells; T1 = tyrphostin AG494; T2 = tyrphostin AG1478; C = curcumin. Statistical significance in relation to control: black font = G2 phase; white font = G1 phase. *$0.01 < P < 0.05$.

Influence of EGFR Inhibitors and Curcumin on LN229 Cell Viability

The investigated tyrosine kinase inhibitors and polyphenol were also examined for their cytotoxic potential and the ability to induce tumor cell apoptosis or necrosis. As a positive control antineoplastic and antimitotic drug, PTX was used. The influence of tested compounds on target cell viability was assessed after double-staining with Hoechst 33342 and propidium iodide. Viable cells were stained on dark blue with oval nuclei, apoptotic cells were stained light-blue, and necrotic cells were stained red. Apoptotic cells were clearly distinguishable by their characteristic morphology (cytoplasmic blebbing, cell shrinkage, nuclear condensation, and fragmentation, the so-called apoptotic bodies; Fig. 6). The results of quantitative determination of

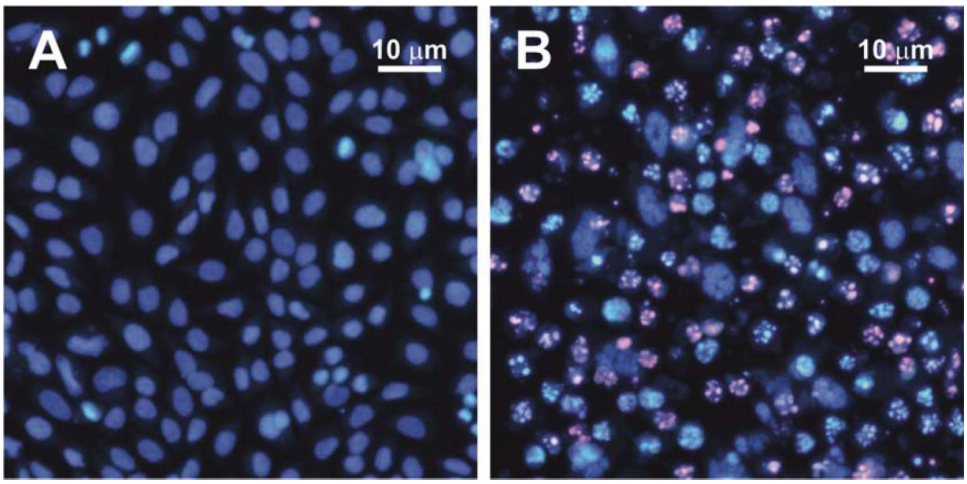

FIG. 6. A typical picture of cultures of control cells (A) and cells treated with mixture of AG494 and curcumin in IC_{50} concentration (B) after 48 h of incubation. Description in the text.

the viability of *Glioblastoma* cells are presented in Fig. 7. Both the investigated receptor kinase inhibitors in IC_{50} concentrations did not affect the viability of LN229 cells. Curcumin, unlike the tyrphostins, in both used concentration significantly induced apoptosis in LN229 cells, in dose–dependent manner. The viability of *Glioblastoma* cells decreased about 20% (IC_{50}) and 50% ($2 \times IC_{50}$) in relative to control. The investigated polyphenol in higher concentration had greater cytotoxic potential than commonly used drug PTX, used in 10 nM concentration ($2 \times IC_{50}$). Addition of tyrphostins to cells treated with curcumin in IC_{50} significantly increased cytotoxic effect of curcumin. The viability of LN229 decreased by 20% (regardless of the tyrphostin used) in relative to polyphenol alone.

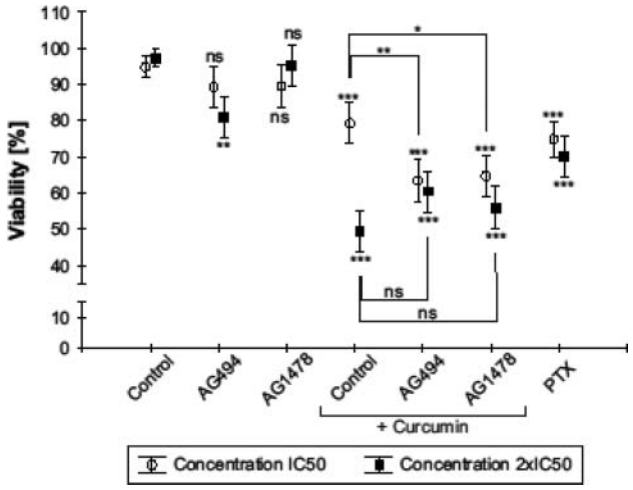

FIG. 7. The effect of tyrphostins: AG494, AG1478, curcumin (C) and its mixtures (PTX = taxol; as the positive control) on the viability of LN229 cells after 48 h of incubation in serum free DMEM/F12+. The tested compounds were used in IC_{50} (transparent dot) or $2 \times IC_{50}$ concentration (black dot). Vertical bars represent 0.95 confidence interval. Statistical significance (in relative to control): $*0.01 < P < 0.05$; $***P < 0.001$.

Activity of Effector Caspases as a Measurement of Proapoptotic Properties of the Investigated Inhibitors

Influence of curcumin and tyrphostins (AG494, AG1478) on the activity of caspases 3/7 on LN229 cells in chemically defined medium (DMEM/F12+) was tested. The cells were exposed to the investigated compounds in $2 \times IC_{50}$ concentrations. As a negative control served cells cultured in analogous conditions in DMEM/F12+ with 1 mM DMSO, whereas the positive control cells were cultured with PTX in concentration 10 nM ($2 \times IC_{50}$). Results are shown in Fig. 8. After 12 h of incubation only AG1478 stimulated activity of 3/7 caspase in statistically significant manner. Other investigated compounds were without effect. Elongation of the incubation time to 24 h showed essential differences in biological response of LN229 cells. Addition of tyrphostins to cells treated with curcumin in IC_{50} significantly increased cytotoxic effect of curcumin. Tyrphostin AG494 and curcumin increased enzymatic activity of the caspases 7× and 16×, respectively. Unexpectedly, the mixtures of both inhibitors were without effect. Similar value of fluorescence determined for curcumin and its mixture with tyrphostin AG1478 indicate that real stimulator of 3/7 caspases activity is curcumin. Presented data indicate that the most effective stimulators of apoptotic process of LN229 cells are curcumin (included curcumin+AG1478) and the mixture of tyrphostins AG494 and AG1478.

Effect of the Investigated Inhibitors on Synthesis and Scavenging of ROS

LN229 cells were exposed to the investigated compounds in concentrations outlined previously as IC_{50} and $2 \times IC_{50}$. As a negative control served cells cultured in analogous conditions in DMEM/F12+. Results are shown in Fig. 9. The experiments on influence of curcumin, tyrphostins, and its mixtures on ROS synthesis indicated that none of the

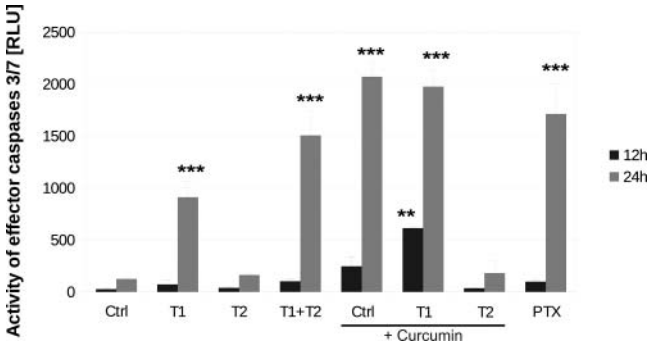

FIG. 8. Activity of caspases 3 and 7 in LN229 cells after 12 and 24 h of incubation with tested compounds and their mixtures used in 2 × IC50 concentration, dissolved in serum-free DMEM/F12+ medium. Each bar is an average of 3 independent experiments. Vertical bars, SD. RLU = relative luminescence unit; Ctrl + DMSO = control cells with solvent of tested compounds; T1 = tyrphostin AG494; T2 = tyrphostin AG1478; C = curcumin; PTX = paclitaxel. Statistical significance in relation to control:): $^{**}0.001 < P < 0.01$; $^{***}P < 0.001$.

investigated compounds was able to stimulate this process (data not shown). In contrast, 2 of 3 investigated inhibitors influenced scavenging ROS induced by hydrogen peroxide treatment in LN229 cells. Tyrphostin AG494 used in concentration IC_{50} and 2 × IC_{50} decreased the level of ROS to 35.5% and 15.8% of control, respectively. Effect of curcumin differs from that obtained for AG494. Only in concentration 2 × IC_{50} this polyphenol decreased the level of ROS to about 40% of control. Tyrphostin AG1478 was practically without effect on scavenging of ROS in LN229 culture. Decrease of scavenging of ROS after treatment with mixture of curcumin and tyrphostin AG494 or tyrphostin AG494 and AG1478 was similar (14–25% of control) and independent on the concentration of the constituents. The effect of AG1478 and curcumin was observed only at concentration 2 × IC_{50} (level of ROS decreased to about 40% of the control). In the case of mixtures, the effect was dependent on the concentration of the

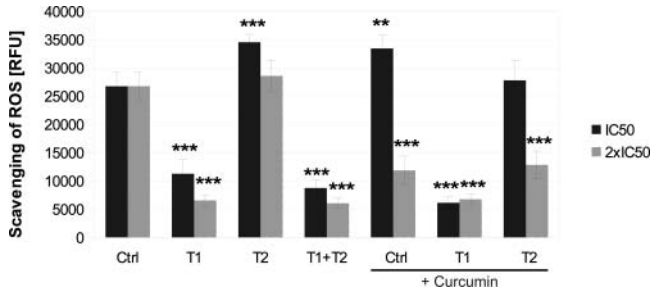

FIG. 9. The effect of tested compounds and their mixtures on scavenging of reactive oxidant species (ROS) after hydrogen peroxide treatment in glioblastoma cells. Cells were incubated with curcumin, tyrphostins, and their mixtures dissolved in DMEM/F12+ medium by 1 h. Each bar is an average of 5 independent experiments. Vertical bars, SD. RFU = relative fluorescence unit; Ctrl+DMSO = control cells with solvent of tested compounds; T1 = tyrphostin AG494; T2 = tyrphostin AG1478; C = curcumin. Statistical significance in relation to control: $^{**}0.001 < P < 0.01$; $^{***}P < 0.001$.

investigated compounds. Decrease of scavenging of ROS after treatment with mixture of curcumin and tyrphostin AG494 or tyrphostin AG494 and AG1478 was independent on the concentration of the constituents, whereas the effect of AG1478 and curcumin was observed only at concentration 2 × IC_{50}. Obtained results indicate that tyrphostin AG494 and its mixtures are most potent scavengers of reactive oxygen species in the culture of LN229 cells.

DNA Damage and Repair in *Glioblastoma* Cells After Treatment with Tested Compounds

The comet assay was performed after 48 h of incubation with tyrphostins and curcumin and their mixtures. The experiment was carried out in 2 series (each was done twice), in which the compounds were used in IC_{50} or $2xIC_{50}$ concentration. The results are presented on Fig. 10. Both tested tyrphostins did not induce the DNA damage in LN229 cells, regardless of the concentration used. Genotoxic properties of curcumin were proportional to the concentration of the compound, but the percentage of DNA damage did not exceed 23% even at 2 × IC_{50} concentration. Mixtures of the curcumin and tyrphostins at 2 × IC_{50} concentration slightly induced DNA damage of tested cells (16–18% in relative to control). However there was no statistical significance in relative to curcumin alone. Therefore DNA damage in *Glioblastoma* cells is probably caused only by the polyphenol. In the case of curcumin–AG1478 mixture at IC_{50} concentration, curcumin slightly (but significantly) induced genotoxicity of tyrphostin (from 10% to 18% in relative to control cells). Although there were no significant differences between genotoxicity of the

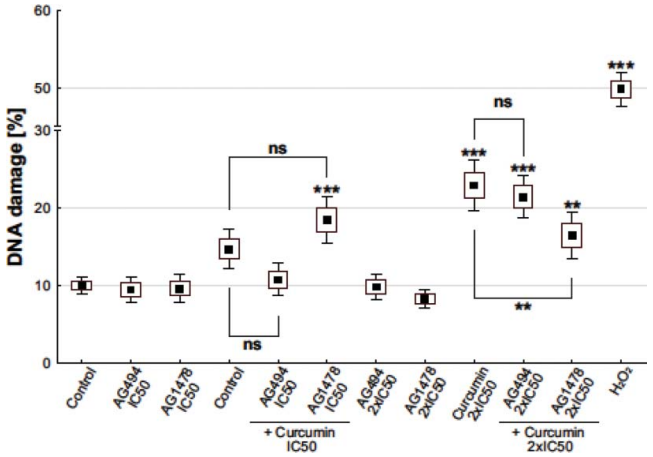

FIG. 10. The level of DNA damage in LN229 cells after 48 h of incubation with inhibitors and their mixtures in concentration of IC50 and 2 × IC50, dissolved in serum free DMEM/F12+ medium. Small square: average; big square: average ± SD; vertical bars: average ± 1.96 * SD. ***DNA damage (%)*** = percentage of the DNA, which migrated to the comet's tail. Statistical significance (in relative to control): $^{*}0.01 < P < 0.05$; $^{***}P < 0.001$.

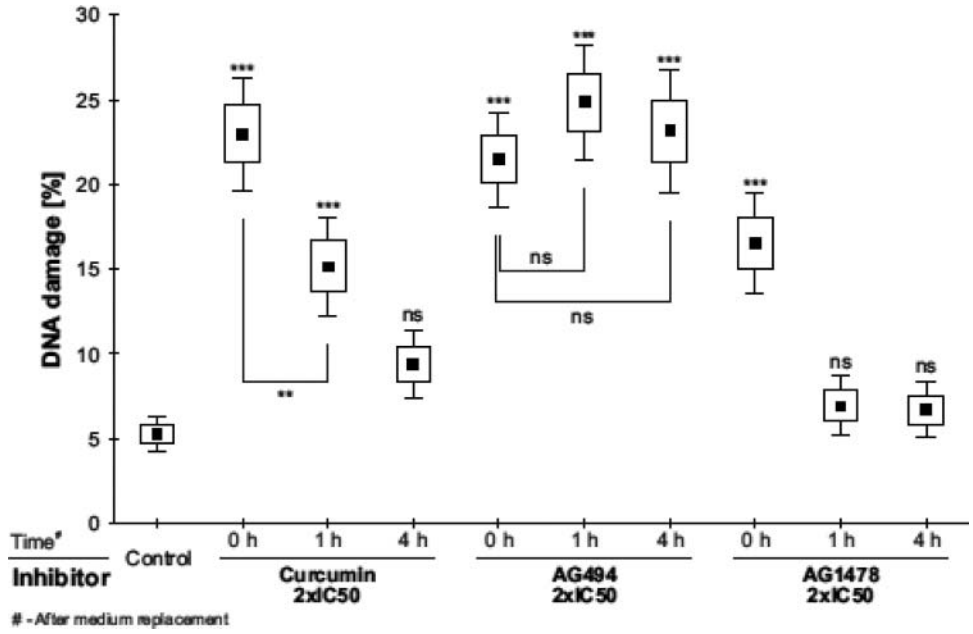

FIG. 11. DNA damage in LN229 cells after 48 h of incubation with curcumin and its mixtures with AG494 and AG1478 used in concentration of $2 \times IC_{50}$, and DNA repair after 1 and 4 h incubation in fresh defined medium, without inhibitors. Black square: an average of 3 independent experiments; big square: average $\pm$ SD; vertical bars: average $\pm$ 1.96 * SD. DNA damage (%) = percentage of the DNA, which migrated to the comet's tail. Statistical significance in relation to control: *$0.01 < P < 0.05$; **$0.001 < P < 0.01$; ***$P < 0.001$. Statistical significance of mixture in relation to compound used alone is marked by connector sign.

curcumin alone and the mixture with tyrphostin, the effect of the mixture was more statistically significant than for polyphenol. In contrary, the mixture of AG494 and curcumin in IC_{50} concentration was not genotoxic to human brain cancer cells.

After evaluation of genotoxic properties of tested compounds, we decided to test whether the DNA damage can undergo repairs. To do that, the cells treated by 48 h with curcumin, tyrphostins, and their mixtures were rinsed with the fresh medium. Then cultures were left for 1 or 4 h in defined medium without inhibitors. Afterwards, cells were trypsynized and comet assay was performed. Results of this experiment are placed at Fig. 11. Both curcumin and its mixtures at concentration $2 \times IC_{50}$ significantly induced the DNA damage in LN229 cells. One-hour incubation in fresh defined medium was sufficient to undergo cellular repairs only in the case of curcumin mixed with AG1478. The DNA damage decreased from 18% to values similar to the control cells. In the case of curcumin at $2 \times IC_{50}$ concentration, such phenomenon was observed only after 4 h of incubation in inhibitors free medium. Moreover, the repairs of damaged DNA were directly proportional to the concentration of the compound. In contrary, curcumin mixed with AG494 caused nonreversible permanent DNA damage in *Glioblastoma* cells. Even after 4 h incubation with fresh inhibitors free medium, the percentage of DNA damage was almost the same as in the case of LN229 cells treated with those mixtures.

DISCUSSION

Advanced clinical studies of alternative methods of *Glioblastoma* treatment have been performed: radio- and chemotherapy with gefitinib or erlotinib and concomitant administration of tyrphostins directed against different receptors (erlotinib with bevacizumab, VEGFR's inhibitor) (18,19). Curcumin, either alone or in combination with other agents, has demonstrated potential against numerous cancer types in human clinical trials (20). That approach strongly emphasized the importance of tyrphostins and curcumin in potential combined anticancer therapy. Although there have been various preclinical studies of antitumor properties of curcumin (21–24) and tyrphostin AG1478 (25,26), studies of curcumin as an adjuvant in brain cancer treatment with tyrphostins have not yet been performed. In this study, we investigated whether curcumin contributes to antitumor properties of tyrphostins AG494 and AG1478. Origin of human malignant gliomas lays in multiple genetic alterations. One of them is widely described amplification of genes, coding the epidermal growth factor receptor and its ligand, leading to their overexpression and coexpression. EGF/EGFR autocrine loop is well documented in approximately 40–60% of glioblastomas (27). The LN229 cells (like as normal tissues or other established GBM cell lines) exhibit overexpression of wtEGFR (19,27), wtPTEN gen, and mutated protein p53. According to the above features, we used that cell line as a model of

EGFR-induced cancer. High levels of EGFR expression have been shown to be correlated with malignant progression in gliomas and associated with a poor prognosis and resistance to therapies (28,29). Therefore, therapeutic strategies directed against the EGFR may have potential in GBM.

The results described in this article indicate that the blockade of EGFR signaling pathways by tyrphostins and curcumin inhibits proliferation of human brain cancer cells in vitro. IC_{50} determined by 2 different methods was compound specific. Tyrphostin AG1478 was a more potent inhibitor of *Glioblastoma* cell growth than AG494 (100× time) and curcumin (40×). The obtained data suggests that the EGF/EGFR autocrine loop induces a specific signalling route that determines the cell's fate. AG1478 and curcumin completely inhibited the growth of LN229 cells, in contrary to AG494. Addition of curcumin to cells treated with tyrphostins in IC_{50} concentration significantly increased cytotoxic effect of AG494. Surprisingly LN229 cells were 10 or 8 times more sensitive to AG1478 than clinically used erlotinib (30) and gefitinib (31), respectively. Although AG494 had similar IC_{50} to that clinically used EGFR's inhibitors (30,31). Differences in kinetics of growth inhibition between used tyrphostins were probably caused by different ability of binding to ATP binding pocket of tyrosine kinase domain in intracellular part of the receptor. IC_{50} coefficient of isolated form of EGFR kinase for AG1478 and AG494 was 3 nM and 1.2 μM, respectively. That implies 400 times higher affinity of AG1478 to bind the tyrosine kinase domain than in case of AG494. Therefore AG1478 used in lower concentrations was stronger cytostatic agent than AG494. Obtained data were similar to our previous work (14), in which AG1478 stronger supressed proliferation of human lung cancer cells (A549) and human prostate cancer cells (DU145) than AG494. AG494 is structurally very similar to polyphenol erbstatin (8,9), what might in some extent explain the similarity of growth inhibition curves of AG494 and polyphenol curcumin.

The cell cycle analysis by flow cytometry showed, that tyrphostin AG1478 in IC_{50} concentration arrested *Gliobastoma* cells in G1/G0 phase of the cycle, which is consistent with the literature (8,9). Mechanism of cytostatic effect of AG1478 was based on cell cycle disruption. Whereas AG494 and curcumin in IC_{50} concentration had no influence on the cell cycle of tested cells. That might imply that their cytostatic properties were based on different mechanism than in case of AG1478. Furthermore it was tyrphostin AG1478 that was responsible for the cell cycle arrest in G1 phase in cultures treated with mixtures of both tyrphostins or with AG1478 with curcumin. In case of mixture of AG494 with curcumin, the effect is quite opposite. The number of cells in the G1 phase was decreased by 20% in relation to control, whereas the number of cells in S phase was increased by 19% in relation to control. This allowed us to draw a hypothesis that cells might be dying during G1/G0, because of cytotoxic properties of the mixture. Therefore accumulation of the cells in S phase of the cycle was artificially observed.

The differential staining method showed that AG1478, regardless of concentration used, had no cytotoxic properties in LN229 cells and AG494 in 2 × IC_{50} slightly reduced their viability. Curcumin, unlike the tyrphostins, in both used concentrations significantly induced apoptosis in *Glioblastoma* cells in a dose-dependent manner. Cytototoxic properties of curcumin are well documented in other human brain cancer cells (21,22), however LN229 seems to be more sensitive. Furthermore, polyphenol in higher concentration had similar cytotoxic potential as the commonly used drug PTX (in concentration 10 nM). PTX was examined as a cytotoxic agent on various human cancer cell lines (32). The addition of curcumin to cells treated with AG494 in IC_{50} significantly increased cytotoxic effect of this tyrphostin, which was supported by cell cycle analysis. Curcumin had a pleiotropic effect on the cells. One of them is induction of apoptosis, by several mechanisms (10,33). Curcumin addition to tyrphostins AG494 (in contrast to AG1478) treated LN229 cells improved the elimination of cancer *cells in vitro*, because this mixture stimulated the apoptose process in the investigate cells, determined by double-staining method as well as by stimulation of 3/7 caspase activity and cell cycle analysis. These results were partially supported by comet assay. Similarly, like in the case of the fluorescence staining method, tyrphostins had no genotoxic properties, which is consistent the literature (8,9), whereas the likely genotoxic effect of curcumin and tyrphostin AG494 was significant and nonreversible. As for cytotoxicity, genotoxicity of curcumin is also well documented in various cell lines (21–23,33). Mechanism underlying in curcumin's genotoxicity was recently described in few papers. Curcumin in low dosage probably induces production of ROS within cells, formation of 8-oxo-deoxyguanosine, and indirect DNA strand breaks (34), whereas polyphenol in high dosage is an reversible inhibitor of topoisomerase II. Distraction of its enzymatic activity may result in chromosome translocations, similar to ones found in different types of leucemia (35,36). Our data provided by DCFH2-DA Assay partially support both mechanisms. In IC_{50}, curcumin induces production of ROS by 30%, whereas in 2 × IC_{50} ROS were scavenged almost by 60%. Observed phenomenon may explain the switch between mechanism of curcumin-induced genotoxity in LN229 cells. However there are reports in which authors demonstrated that curcumin induces apoptosis in DNA-damage independent manner (37), drives cells to senescence rather than leads to apoptosis (38), arrests cell cycle after oxidative stress (39), does not have genotoxic potential at all (40) or even inhibits DNA-damage induced by oxidative stress (41). However what is important is that results of polyphenol treatment are highly dependent on type of investigated cell line and concentration of curcumin. Nonetheless it is difficult to distinguish whether observed accumulation of DNA breaks leaded to cell death or was a result of a late apoptosis of LN229 cells after treatment with

tested compounds. Therefore in our study, we assume that curcumin induced genotoxity in *Glioblastoma* cells, yet it needs to be confirmed with other more specific tests (immunoF-based 53BP1 and γH2AX detection or activation of proteins involved in DNA damage response pathway). These results were supported by cell cycle analysis by flow cytometer. Tyrphostin AG1478, regardless of concentration used, acts like a strong cytostatic agent, which arrests cells in G1/G0 phase, unlike AG494 and curcumin and their mixtures, which were strong cytotoxic agents in *Glioblastoma* treatment. These compounds stimulate LN229 cells to go through apoptotic pathway rather than arrest their cell cycle. However level of DNA damage was 2 times lower, than decrease of viability. Both methods were applied to measure the proapoptotic effect of investigated compounds, but they are based on different assumptions, hence differences in obtained results. On the other hand, these differences may result from long incubation (48 h) with curcumin and its mixtures, during which molecular mechanism of DNA repair could work within cells. This was proved, by evaluation whether the DNA damage caused by tested compounds could be reversed. In case of curcumin, the DNA repair is time dependant and complete. Furthermore this is consistent with literature (35,36), in which it is stated that curcumin in high concentrations act like reversible topoisomerase II inhibitor. However observed phenomenon might by partially explained by selection of resistant cells (described below). High cytotoxic and probably minor genotoxic potential of curcumin support the hypothesis that curcumin in IC_{50} overall changes kinetics of LN229 cell proliferation. After removal of polyphenol from growth medium, only resistant cells, which survived the treatment, proliferated in clonal way. AG494 had a minor proapoptotic effect in $2 \times IC_{50}$, hence this phenomenon was not so strongly observed, however reduction of proliferation rate after removal of tyrphostin was dose dependent. Combining these two features, it can be hypothesized why cells treated with curcumin and AG494 did not undergo the DNA repair. The DNA damage was so potent, reflecting the apoptosis that most cells probably died. These results were strongly supported by photographs from fluorescent microscopy. The late apoptosis (presence of apoptotic bodies) as well as dead cells (marked red) after treatment with used compounds clearly imply that observed phenomenon is not reversible, but it leads to cell death. Thus in this case curcumin-induced DNA damage do not trigger DNA repair and that is why apoptotic processes within treated cells can be observed. Therefore even after 4 h in inhibitor free medium the DNA repair was not observed (in relative to control cells and treated cells). In contrary, AG1478, which had no cyto- and genotoxic properties, only arrested cells in G1/G0 phase of cell cycle, inhibited the growth of brain cancer cells in completely reversible manner.

Cotreatment of curcumin with AG1478 and AG494 caused the greater growth inhibition than each inhibitor treatment alone. These results were supported by Hui-Ping Lin et al. (42). In this work, low dosage cotreatment of curcumin with AG1478 significantly suppressed growth of the 2 lung cancer cell lines. That might imply that curcumin could be an adjuvant in small molecule inhibitors' treatment, regardless of cell type. The explanation of greater growth inhibition of cotreatment with curcumin (number of cells decreased below their initial number) probably is the ability of curcumin to significantly enhance the proapoptotic properties of EGFR inhibitors (as described above). It is most interesting that 4–6 μM curcumin alone inhibited the proliferation of LN229 cells only by 20%, but combination of curcumin and AG1478 abolished the inhibitory effect of tyrphostin by 15%. That phenomenon is widely described as a *hormetic effect* (33): low doses of curcumin could act like a protective agent, whereas in high doses it could act like cytostatic, cytotoxic, and genotoxic agents. This is not an isolated case, such a phenomenon is observed also for other polyphenols, like quercetin (43).

Studies performed by our research group had 2 main drawbacks. Firstly, LN229 cell line was not the ideal model of *Glioblastoma* disease in vitro. None of the established human brain cancer cell lines express the constitutively active mutant variant of EGF receptor (EGFRvIII) (19). Although it is widely observed that in 25% of all GBMs (19) and 60–70% of EGFR-overexpressing GBMs (30), cells coexpress wild type EGFR and EGFRvIII, as well as other mutations in EGFR (27). However, used by us, tyrphostin AG1478 has higher affinity for mutant forms of the receptor (e.g., EGFRvIII) than the wild type form (44). Therefore this tyrphostin might be a stronger inhibitor of growth of brain tumors than clinically used erlotinib (EGFR expression needed to response to inhibitor) (45) and similar to gefitinib (EGFRvIII expression needed to response) (30). Importantly, tyrphostin AG1478 was examined in mice bearing xenografts of the human glioblastoma cell line U87MG.D2–7, which expresses a constitutively active variant of the EGF receptor (25). In this study autophosphorylation and downstream signaling was inhibited in a dose-dependent manner by injection of AG1478 and dosage regiment in rodents was established. Secondly, curcumin is poorly absorbed by intestinal cells (low aqueous solubility and stability), rapidly metabolized by liver, and rapidly eliminated from system (33). Moreover concentrations that were commonly used in in vitro studies were frequently higher than those that might be achievable in physiological conditions. The highest achieved serum level of the curcumin was up to 2 μM, 1 h after administration, during its oral ingestion of 8000 mg per day (46). Although the Hindu people, who are in the nation with the highest daily intake of curcumin (present in the turmeric spice), consume up to 100 mg/day of active substance (47). Therefore potential therapeutic use of that polyphenol might be questionable due to its limited bioavailability. However numerous studies have been performed to redress these crucial issues [e.g., co-administration with piperine, the active substance from pepper, which can increase the curcumin's level in the blood even 30 times (48), increased aqueous solubility and stability by conjugation

with alginate (49) or improved cell targeting by self-assembling peptide nanofiber carrier (50)]. Furthermore clinical trials showed that even extremely high daily doses of curcumin intake (12 g/day) were harmless to the patients (46).

After oral administration of the curcumin, it is metabolized to tetrahydrocurcumin by the alcohol dehydrogenase and then translocated to the bloodstream. This process is crucial to induce both local and systemic effects and it is mediated by erythrocytes. Red blood cells allow curcumin's derivatives to reach the specific receptors on surface of target cells. However studies have shown that exposure of erythrocytes to curcumin (in concentration about 1 uM) enhances eryptosis, the suicidal death of erythrocytes (51). Thus changes in red blood cells lifespan may represent a dose-limiting sign of curcumin's toxicity. The result of eryptosis is phosphatidylserine exposure at the red blood cells surface. This in turn leads to phagocytosis by macrophages and liver Kupffer cells and therefore elimination from the circulating blood (52). Degradation of dead erythrocytes by phagocytic cells, stimulates them also to local O_2 production, which, by activating NF-kB and JNK, leads to enhanced production of proinflammatory cytokines like TNFα and IL-6. These are characteristic for metabolic syndrome and represent only few of metabolic risk factors for atherosclerotic-cardiovascular diseases (ACVD) (53).

Our investigation demonstrated that combined treatment with a low dosage of curcumin (7–14 μM) with a relatively low concentration (12–24 μM) of tyrphostin AG494 against EGFR dramatically reduced growth and viability of LN229 cells. These concentrations of curcumin and inhibitor are possible to achieve in patient serum via oral or injection administration. Furthermore cotreatment with low dosage curcumin and investigated tyrphostins had signifcantly higher proapoptotic potential than each inhibitor used alone. In conclusion, combined treatment with a low dosage of curcumin with therapies against EGFR may be an adjuvant therapy beneficial to GBM patients.

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