# *In vitro* novel combinations of psychotropics and anti-cancer modalities in U87 human glioblastoma cells

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**Abstract.** Glioblastoma multiforme (GBM) is a highly aggressive malignant brain tumor. Despite some recent improvement in the treatment of this malignancy, life expectancy of GBM patients remains extremely low. Therefore, continuous efforts to develop new treatment modalities are mandatory. A novel approach to cancer treatment is the use of targeted treatments, alone and in combination with other therapies. In this study, we evaluated the effects of novel combinations of conventional anti-cancer treatments (temozolomide or irradiation) with the targeted drug, imatinib, or with psychotropic drugs, belonging to the selective serotonin reuptake inhibitors (SSRIs) and phenothiazine subclasses, as well as combination of imatinib with psychotropic agents, on a human U87 glioblastoma cell line. The combination of temozolomide with imatinib or the psychotropic drugs resulted in an additive anti-proliferative effect, while the combination of irradiation and the psychotropic agents resulted in a less than additive effect on cell proliferation. A marked synergistic anti-proliferative effect of imatinib combined with the psychotropic drugs fluoxetine, sertraline or perphenazine was demonstrated. None of the single or combined treatments led to a reduction in the expression of phosphorylated MAP kinase. However, a marked synergistic reduction in the expression of the key regulatory molecule, pAKT, was detected, following the combined treatment of the cells with the imatinib/psychotropics combination. This down-regulation of pAKT may mediate the synergistic anti-proliferative interaction of imatinib with the psychotropic agents. Although the concentrations of the psychotropic agents used in this and other *in vitro* studies were beyond the

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clinically relevant blood levels in humans, recent studies have demonstrated anti-proliferative effects *in vivo*, using sertraline in a human colon cancer model. Thus, it seems that further *in vivo* studies combining imatinib with psychotropic agents, especially fluoxetine and sertraline, are warranted.

## Introduction

Glioblastoma multiforme (GBM) is a highly aggressive malignant brain tumor. GBM is a genetically polymorphic malignancy with a variety of deletions, amplifications and point mutations. These molecular changes lead to the activation of signal transduction pathways downstream of tyrosine kinase receptors, such as epidermal growth factor receptor (EGFR), or platelet-derived growth factor receptor (PDGFR) (1,2).

Despite some recent improvement in the treatment of this malignancy using the combined treatment of temozolomide (temodal) and irradiation (3,4), the life expectancy of GBM patients remains extremely poor (3,4). Therefore continuous efforts to develop new treatment modalities are needed. One novel approach to cancer treatment is the use of targeted rationalized treatments, directed towards key regulators of cell proliferation and apoptosis, alone and in combination with other treatments. Membranal receptors and intracellular proteins with tyrosine kinase activity involved in the cellular signal transduction pathways are the main targets for the development of novel anti-tumor agents.

Imatinib mesylate (Gleevec®; STI571) was developed against the tyrosine kinase activity of the mutated *BCR-ABL* gene product in chronic myeloid leukemia and proved to be highly effective against this type of leukemia. Subsequently, imatinib was found to inhibit the tyrosine kinase activity of stem cell factor (KIT), platelet-derived growth factor (PDGF), colony stimulating factor (CSF-1R) and discoidin domain (DDR) receptors and to be effective in the treatment of gastro-intestinal stromal tumors (GIST) (5-8). GBM is characterized by the overexpression of the PDGFR as well as EGFR (9), which signal through RAS and protein kinase C (PKC) (9). U87 human glioblastoma cell line which overexpresses PDGFR and EGFR serves as an *in vitro* cellular model for glioblastoma (10).

A recent report suggested that possible combinations of anti-PDGFR and EGFR agents with other treatment modalities could provide a better alternative for the treatment of GB tumors compared with monotherapy (11-15). Previous studies from our and other laboratories (16-19) have demonstrated the anti-tumor effect of psychotropic agents, such as antidepressants and antipsychotics. Our laboratory demonstrated that sigma receptor ligands, mainly haloperidol, possess pronounced anti-tumor activity. In our previous study, the combination of haloperidol and imatinib was found to exert a marked synergistic anti-proliferative effect on the SKMEL 28 human melanoma cell line (17).

In this study, we evaluated the effects of conventional combinations, as well as novel combinations on the human U87 glioblastoma cell line. We examined the *in vitro* effects of irradiation combined with temozolomide (the currently preferred treatment for human GBM), on cell proliferation. Novel combinations examined including treatment of U87 cells with the PDGF-targeted therapeutic agent, imatinib, combined with several psychotropic agents, belonging to the selective serotonin reuptake inhibitors (SSRIs) or phenothiazines. We assessed cell proliferation, intracellular ATP content and the phosphorylation state of AKT and mitogen-activated protein kinase (MAPK), key molecules implicated in the cellular effects of both imatinib and psychotropic agents (20-23).

## Materials and methods

*Materials*. Imatinib (kindly provided by Novartis Pharmaceuticals, Basel, Switzerland), fluoxetine (Unipharm, Ramat-Gan, Israel), sertraline (Ferring Pharmaceuticals, USA) and temozolomide (Schering-Plough Corporation, Germany) were dissolved in distillated water (10 mM stock) and kept frozen for up to 6 weeks. Perphenazine (Sigma, Israel) was dissolved in 1% lactic acid (10 mM stock) and kept frozen for up to 6 weeks. Sulphorhodamine B, Tris base, propidium iodide, trypan blue were obtained from Sigma.

*Cell culture*. U87 human glioblastoma cell line (kindly provided by Dr Gad Lavie, The Sheba Medical Center, Ramat-Gan, Israel) was maintained in DMEM (4.5 mg/l D-Glucose), supplemented with 10% fetal calf serum, 2 mM glutamine and antibiotics (penicillin and streptomycin) which were obtained from Biological Industries (Beit Haemek, Israel). Cells were incubated at 37˚C, in a humidified atmosphere of 5% $CO_2$ and 95% air.

*Cell proliferation*. Cells ($1x10^4$/ml) were seeded in quadruplicates in 24 well-plates in the presence or absence of several concentrations of the tested drugs alone or in combination. Imatinib was combined with each one of the following psychotropic drugs: fluoxetine, perphenazine, sertraline or temozolomide. Temozolomide was combined with perphe-nazine or sertraline. Cells were cultured for seven days and proliferation was assessed by the SRB assay (24). Briefly, cells were fixed with 10% trichloroacetic acid and stained with 0.4% sulphorhodamine B dissolved in 1% acetic acid. Unbound dye was removed by four repeated washes with acetic acid (1%), and the dye-stained protein was extracted with unbuffered Tris base (10 mM). The absorbance of the stained protein samples at 550 nm was determined, using a 96 well microtiter ELISA plate reader (PowerWave X, Bio Tek Instruments, Inc., USA).

The inhibitory effect of each agent added to the cultures was calculated as follows: Inhibition (%) = [1- (OD of treated wells/OD of control wells)] x100. The theoretical additive inhibitory effect of two agents (a and b) was calculated as described in (25,26), using the following equation: $I$ab = 100x [1-(1- $I$a/100) x (1- $I$b/100)] where $I$ab is the calculated additive inhibitory effect expressed as % inhibition. $I$a and $I$b are the measured inhibitory effect (%) of each agent acting alone as compared with that of the control cultures. This equation was derived, assuming that the inhibitory agents act independently on the same target population.

*Irradiation*. Cells ($1x10^4$) were seeded in quadruplicates in 24 well-plates in the presence or absence of 5, 7 $\mu$M perphenazine or 5, 7.5 $\mu$M sertraline. Cells were irradiated 1 h prior, 1 h after, or 24 h after the addition of the drugs, at radiation doses increasing from 0 to 8 Gy, using a 6MV linear accelerator (Varian 600C, Palo Alto, CA, USA). Cell proliferation was assessed seven days after seeding by the SRB colorimetric cytotoxicity assay.

*Cell cycle and apoptosis analysis*. Cells ($5x10^5$/10 ml or $3x10^5$/10 ml) were seeded for 2 or 5 days, respectively with 5 $\mu$M perphenazine or 7.5 $\mu$M sertraline or 15 $\mu$M fluoxetine alone or with 10 $\mu$M imatinib. Floating and adherent cells were combined, washed with PBS and nuclei were prepared from $5x10^5$-$1x10^6$ cells for the flow cytometric analysis, using trypsin, followed by staining of the nuclei with propidium iodide (27). DNA content was analyzed by FACSCalibur (Becton-Dickinson, San Jose, CA, USA), using ModFitLT cell cycle analysis software (Verity Software House Inc., Topsham, ME, USA).

Cells defined by FACS analysis in the pre-G1 stage of the cell cycle were suggested as possibly apoptotic. Apoptosis was also assessed by detecting DNA fragmentation using the Cell Death Detection ELISA[Plus] kit (Roche, Mannheim, Germany). U87 cells ($1x10^4$) were seeded in each well of a 24-well plate. Cells were treated with 10 $\mu$M imatinib, 5 $\mu$M perphenazine, 7.5 $\mu$M sertraline or 15 $\mu$M fluoxetine alone or in combination with 10 $\mu$M imatinib with each one of the psychotropic drugs. After 5 days, 0.25 ml lysis buffer was added to each well and the supernatant was assayed for DNA fragments according to the manufacturer's instructions. The absorbance at 405 and 492 nm was measured using a microtiter ELISA plate reader. Identical replicate plates were analyzed for the estimation of cell number, using the SRB method. The results obtained from the DNA fragmentation assay were then normalized for cell numbers. Results were expressed as relative apoptosis to untreated cells (apoptotic index).

*Determination of AKT and phosphorylated AKT (pAKT) protein expression*. Cells ($5x10^5$/10 ml) were cultured without or with 10 $\mu$M imatinib, 5 $\mu$M perphenazine, 7.5 $\mu$M sertraline or 15 $\mu$M fluoxetine alone or in combination of 10 $\mu$M imatinib with each one of the psychotropic drugs. After 24 h, cells were harvested, washed with PBS and lysed using the
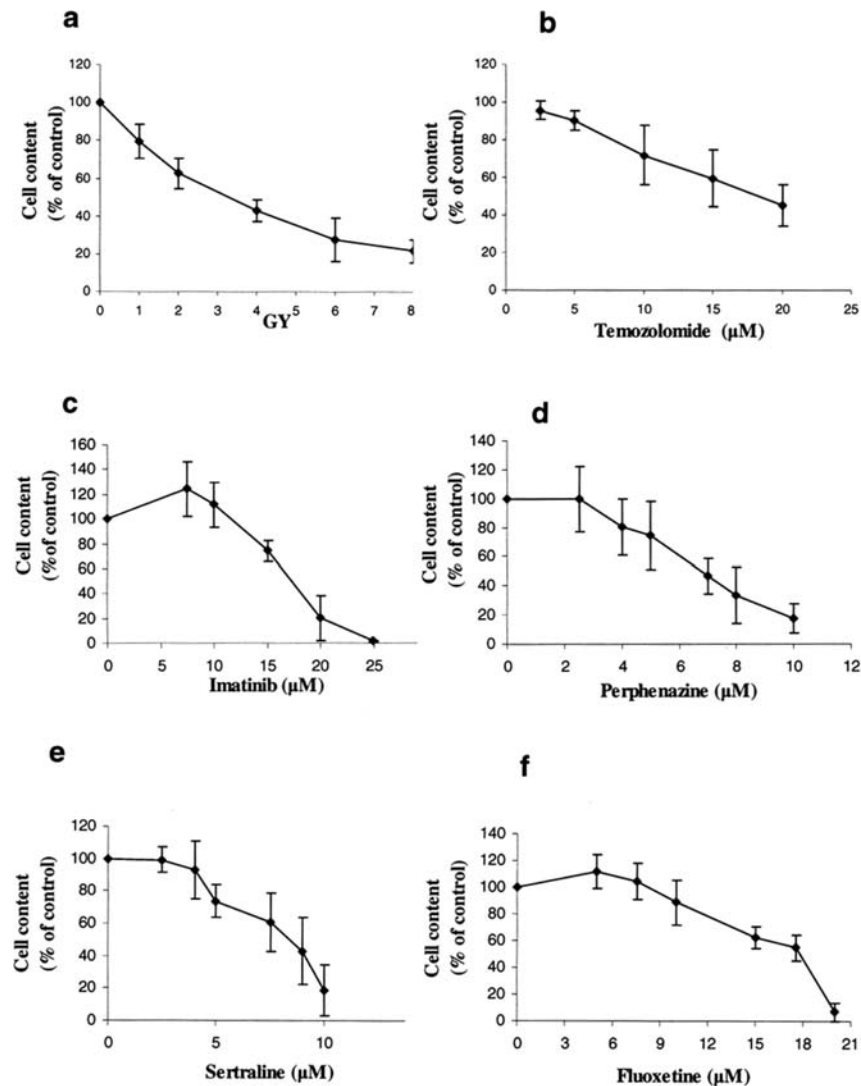
Figure 1. The effects of irradiation, temozolomide, imatinib and psychotropic drugs on U87 glioblastoma cell proliferation. U87 cells (1x10⁴/ml) were treated with (a) irradiation, (b) temozolomide, (c) imatinib, (d) perphenazine, (e) sertraline or (f) fluoxetine for 7 days as described in Materials and methods. Adherent cells were assessed using the SRB assay. Results are expressed as mean ± SEM of at least 3 independent experiments performed in quadruplicates.

TRAP kit CHAPS lysis buffer, containing phosphatase inhibitors. Protein concentration was determined using the Bradford assay (Bio-Rad Labs., Hercules, CA, USA). Identical protein amounts (20 $\mu$g) of all samples were subjected to 10% SDS-PAGE. The AKT protein or the phosphorylated form of AKT protein were detected by specific polyclonal antibodies (Cell Signaling, SC, USA) in 1:1000 dilution, followed by HRP-conjugated goat anti-rabbit antibody (Jackson Lab., West Grove, PA, USA). ß-actin was detected by monoclonal antibody (MP Biomedical, OH, USA) in 1:10,000 dilution, followed by HRP-conjugated goat anti-mouse (Jackson Lab., West Grove, PA, USA). The protein bands were detected by using a chemiluminescent kit (Sigma, USA) and quantified with the VersaDoc™ Imaging System (Bio-Rad) using the Multi Analyst software.

*Determination of MAPK and phosphorylated MAPK (pMAPK) protein expression.* Cells (1x10⁶/10 ml) were cultured for 24 h without or with 10 $\mu$M imatinib or 15 $\mu$M fluoxetine alone or

in combination. Total cellular protein extracts were prepared (50 $\mu$g) and separated as described above. MAPK (ERK 1 and 2) protein was detected using a rabbit polyclonal antibody (1:10,000 Sigma, St. Louis, MO, USA) followed by IRDye™ 680 conjugated goat anti-rabbit IgG (1:10,000 LI-COR, USA) and the phosphorylated form was detected using a mouse monoclonal antibody (1:10,000 Sigma) followed by IRDye™ 800 conjugated goat anti-mouse IgG (1:10,000 LI-COR, USA). Images were acquired with the Odyssey infrared imaging system (LI-COR, Lincoln, NB, USA) and analyzed by the software programs specified in the Odyssey manual.

*Caspase 3-like activity.* Cells (1x10⁶/10 ml) were cultured for 24 h and then exposed to 5 $\mu$M perphenazine, 7.5 $\mu$M sertraline or 15 $\mu$M fluoxetine combined with 10 $\mu$M imatinib for 4,8,12 or 24 h. Adherent and non-adherent cells were collected and washed with PBS and the cell pellets were suspended in 120 $\mu$l lysis buffer (28). After 30 min on ice, cell extracts were centrifuged for 6 min at 8900 g. The supernatants were
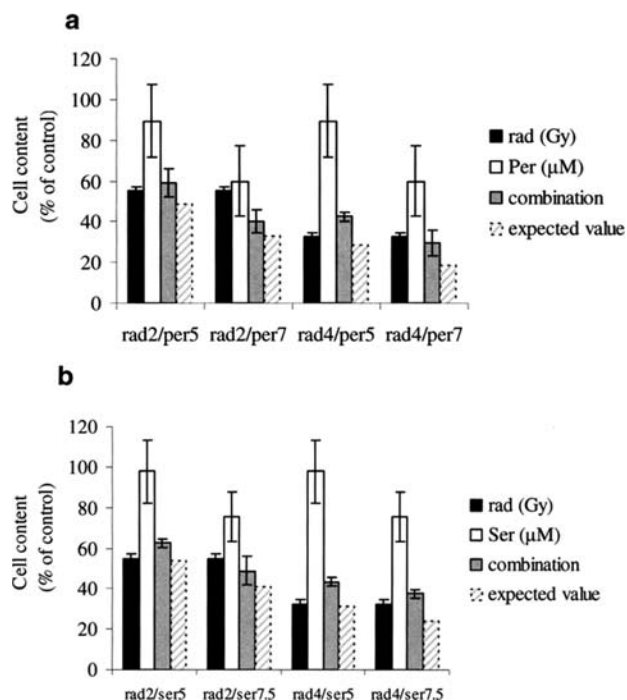
Figure 2. The effect of irradiation treatment combined with perphenazine or sertraline on U87 cell content. U87 cells (1x10⁴/ml) were seeded with (a) perphenazine (per) or (b) sertraline (ser). Twenty-four hours after, the cells were irradiated (rad) and incubated for additional 6 days. Proliferation was assessed using the SRB assay. Results are expressed as mean ± SEM of at least 3 independent experiments performed in quadruplicates. The fourth bar in each section represents the expected calculated additive effect of the combined treatments on cell content.



Figure 3. The effect of temozolomide treatment combined with perphenazine or sertraline on U87 cell content. U87 (1x10⁴/ml) cells were treated with temozolomide combined with (a) perphenazine or (b) sertraline for 7 days as described in Materials and methods. Proliferation was assessed using the SRB assay. Results are expressed as mean ± SEM of at least 3 independent experiments performed in quadruplicates. The fourth bar in each section represents the expected calculated additive effect of the combined treatments on cell content.

collected and frozen immediately at -70˚C for at least 48 h. Protein concentration was determined using the Bradford assay (29). Enzyme activity was determined using the fluorogenic substrate Ac-DEVD-AMC (Alexis Biochemical, Farmungdale, NY, USA), as previously described (28). Specific caspase activity was expressed as the ratio between the reaction rate (increase in fluorescence over time) and protein content.

*ATP measurement*. U87 cells (5x10⁵/10 ml) were cultured in the presence or absence of 10 $\mu$M imatinib, 5 $\mu$M perphenazine, 7.5 $\mu$M sertraline or 15 $\mu$M fluoxetine alone, or in combination with 10 $\mu$M imatinib for 4 or 24 h. The cells were trypsinized, and viable cells were counted using the trypan blue exclusion test. Cells (5x10⁴) were used. After washing, a lysing reagent was added to release intracellular ATP. A luciferin/luciferase mixture (part of ImmuKnow-Immune Cell Function Assay kit, CYLEX incorporated, MD, USA) was then added to the cell lysates. Within 10 min after addition of the enzyme, the bioluminescent product was measured using the GloRunner microplate luminometer (Turner BioSystems, Sunnnyvale, CA, USA). The amount of the light emitted (emission maximum 562 nm) was compared to a calibration curve generated with ATP calibrators (0-1000 ng/ml), and the ATP concentration in each sample was calculated using the ImmuKnow™ Data Analysis Software.
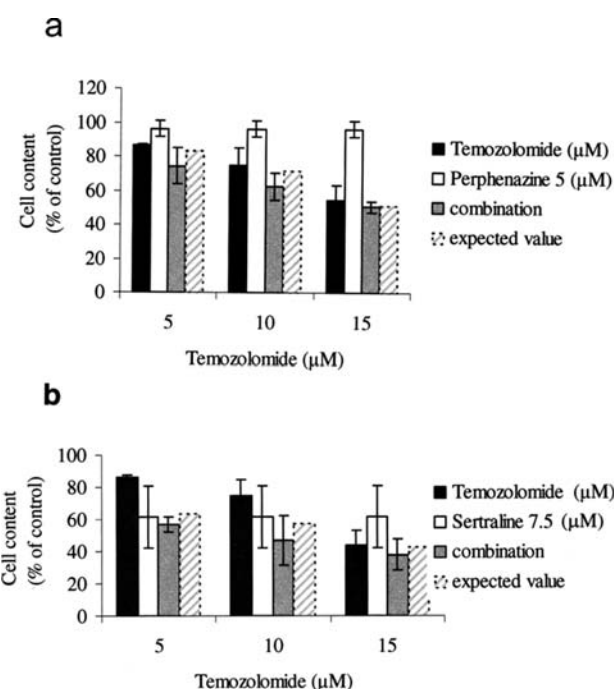
*Statistical analysis*. The data are presented as mean ± SD, or mean ± SEM. Each experiment was performed at least 3 times. The comparison between the groups was analyzed using One-way analysis of variance (ANOVA), followed by Bonferroni post-hoc multiple comparisons test, or Student's t-test, as appropriate. A $p < 0.05$ was considered statistically significant.

**Results**

*The effects of irradiation, temozolomide, imatinib and psychotropic drugs on U87 glioblastoma cell proliferation.* In this study we combined conventional treatment modalities for glioblastoma: irradiation or the chemotherapeutic agent temozolomide, with the tyrosine kinase inhibitor imatinib, or the psychotropic drugs, perphenazine, sertraline, or fluoxetine. Exposure of U87 human glioblastoma cells to increasing doses of irradiation (0-8 Gy), or to increasing concentrations of each of the following agents: temozolomide (0-20 $\mu$M), the tyrosine kinase inhibitor imatinib (0-25 $\mu$M) or the psychotropic agents perphenazine (0-10 $\mu$M), sertraline (0-10 $\mu$M), fluoxetine (0-20 $\mu$M) for 7 days, resulted in a dose-dependent reduction in cell content (Fig. 1a-f). The LD$_{50}$ for irradiation was 4 Gy and the LC$_{50}$ for the different treatments were as follows: temozolomide: 18 $\mu$M, imatinib: 17.4 $\mu$M, perphenazine: 6.8 $\mu$M, sertraline: 8 $\mu$M and fluoxetine 19 $\mu$M, respectively.
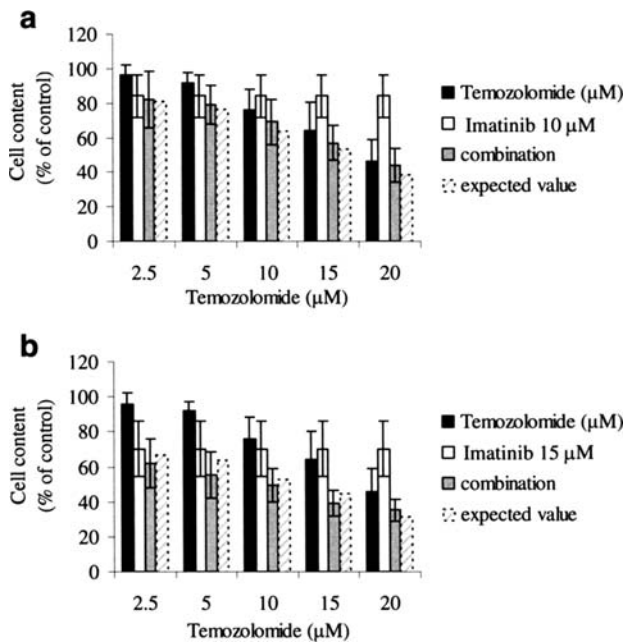
Figure 4. The effect of temozolomide combined with imatinib on U87 cell content. U87 cells (1x10⁴/ml) were treated with temozolomide combined with (a) 10 μM imatinib or (b) 15 μM imatinib for 7 days as described in Materials and methods. Proliferation was assessed using the SRB assay. Results are expressed as mean ± SEM of at least 3 independent experiments performed in quadruplicates. The fourth bar in each section represents the expected calculated additive effect of the combined treatments on cell content.

*The effects of combined treatments on U87 glioblastoma cell proliferation.* In order to test the combined treatments, we used the dose of irradiation or the drug concentrations that inhibited cell proliferation by 50% or less.

*The effect of combined treatments of irradiation with perphenazine or sertraline on U87 cell content.* Fig. 2 shows the effect of the combination of irradiation with (a) perphenazine or with (b) sertraline, on cell content. These combinations of treatments resulted in a less than additive decrease in cell content, regardless whether perphenazine or sertraline were added 1 h before, immediately before or 1 h after irradiation (data not shown).

*The effect of combined treatments of temozolomide with perphenazine or sertraline on U87 cell content.* U87 cells were incubated with 5, 10 or 15 μM temozolomide, combined with 5 μM perphenazine or 7.5 μM sertraline for 7 days. An additive decrease in cell content was observed in all combinations (Fig. 3a and b).

*The effect of combined treatment of temozolomide and imatinib on U87 cell content.* As shown in Fig. 4a and b exposure of U87 cells to increasing concentrations of temozolomide (0-20 μM) combined with 10 or 15 μM imatinib for 7 days, resulted in an additive reduction in cell content.

*The effect of combined treatments of imatinib with perphenazine, sertraline or fluoxetine on U87 cell content.* We tested the effect of the combined treatments of imatinib with three psychotropic drugs: perphenazine, sertraline or fluoxetine. Fig. 5a-c shows that all the combinations decreased the cell content in a synergistic manner. Combined treatment was markedly and significantly more effective in reducing cell content, as compared to each individual treatment, or to
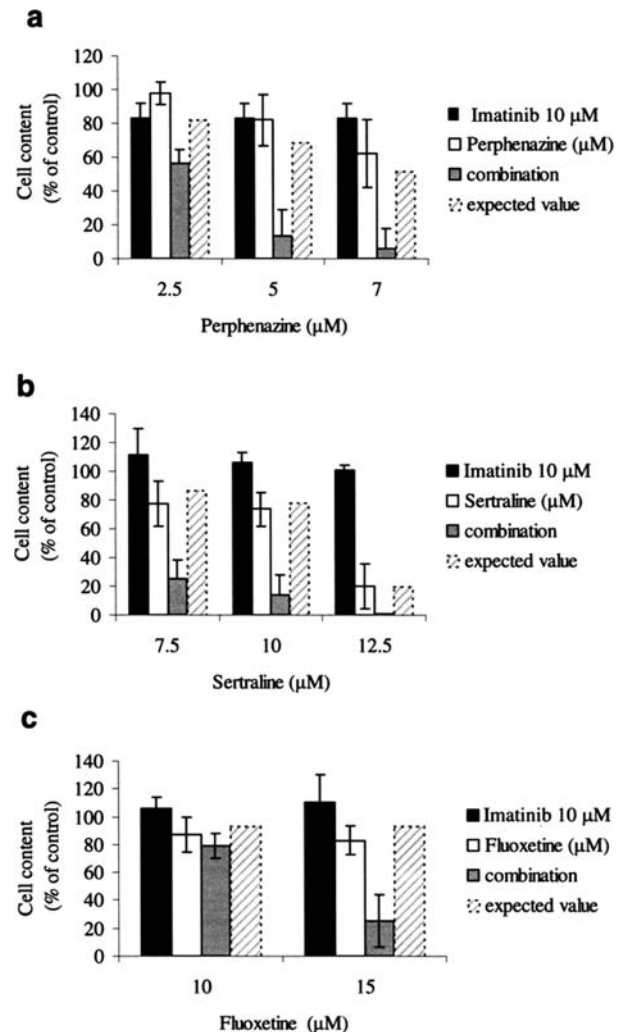


Figure 5. The effect of imatinib treatment combined with perphenazine, sertraline or fluoxetine on U87 cells. U87 cells (1x10⁴/ml) were treated with imatinib combined with (a) perphenazine or (b) sertraline or (c) fluoxetine for 7 days as described in Materials and methods. Proliferation was assessed using the SRB assay. Results are expressed as mean ± SEM of at least 3 independent experiments performed in quadruplicates. The fourth bar in each section represents the expected calculated additive effect of the combined treatments on cell content. In the ANOVA test and independent samples t test, the following abbreviations were used: im, imatinib; per, perphenazine; imp, imatinib + perphenazine; ser, sertraline; ims, imatinib + sertraline; flu, fluoxetine; imf, imatinib + fluoxetine. $F_{(7,50)}=6.674$ 5(a) im10 vs. per2.5 (p=0.131), imp vs. per2.5 (p=0.001), imp vs. im10 (p=0.014), t(df=6) = -3.301, p<0.005 observed vs. expected $F_{(2,9)}=35.346$ im10 vs. per5 (p=0.998), imp vs.per5 (p=0.002), imp vs. im10 (p=0.002), t(df=6) = -8.043, p<0.005 observed vs expected $F_{(2,9)}=31.052$ im10 vs. per7 (p=0.310), imp vs. per7 (p=0.016), imp vs. im10 (p<0.001), t(df=3.09) = -5.639 , p<0.005 observed vs. expected 5(b).im 10 vs. ser7.5 (p=0.086), ims vs. ser7.5 (p=0.007), ims vs. im10 (p=0.001), t(df=6) = -4.444, p<0.005 observed vs. expected $F_{(2,6)}=49.114$ im10 vs. ser10 (p=0.070), ims vs.ser10 (p=0.0017), ims vs. im10 (p=0.007), t(df=4) = -5.244, p<0.005 observed vs. expected $F_{(2,6)}=98.123$ im10 vs. ser12.5 (p=0.028), ims vs.ser12.5 (p=0.421), ims vs. im10 (p=0.001), t(df=2.0) = -2.153, p>0.005 observed vs. expected $F_{(2,9)}=23.733$ (c). im10 vs flu15 (p=0.217), imf vs. flu15 (p=0.010), imf vs. im10 (p=0.004), 5(df=6) = -4.021, p<0.005 observed vs. expected $F_{(2,6)}=5.301$ im10 vs. flu10 (p=0.298), imf vs. flu10 (p=0.827), imf vs. im10 (p=0.062), t(df=4) = -1.035, p>0.005 observed vs expected.

the expected additive interaction between the drugs. Similar results were obtained when imatinib and the corresponding psychotropics were added to the cell culture at the same time,

Table I. The effect of imatinib alone and in combination with psychotropic agents on sub G1 fraction in U87 cells.

| Treatment | Sub-G1 fraction (%) | |
|---|---|---|
| Duration | 2 days | 5 days |
| Untreated cells | 12.3±1.7 | 11.3±1.4 |
| Imatinib 10 $\mu$M | 13.0±2.4 | 12.4±1.8 |
| Perphenazine 5 $\mu$M | 12.4±1.9 | 16.7±4.1 |
| Sertraline 7.5 $\mu$M | 14.3±2.4 | 15.6±1.9 |
| Fluoxetine 15 $\mu$M | 15.3±2.1 | 15.5±2.3 |
| Imatinib 10 $\mu$M + Perphenazine 5 $\mu$M | 21.0±3.7 | 20.6±2.5 |
| Imatinib 10 $\mu$M + Sertraline 7.5 $\mu$M | 23.2±3.5 | 25.1±5.4 |
| Imatinib 10 $\mu$M + Fluoxetine 15 $\mu$M | 24.8±1.1 | 48.1±6.6[a] |
| ANOVA | $F_{(7, 71)}=3.18, p<0.05$ | $F_{(7, 87)}=10.63, p<0.0001$ |

Cells were incubated for 2 or 5 days and cell cycle was assessed as described in Materials and methods. The results represent means ± SEM of 5 independent experiments performed in duplicates. [a]$p<0.05$ vs. untreated, imatinib, fluoxetine, or imatinib + perphenazine groups.
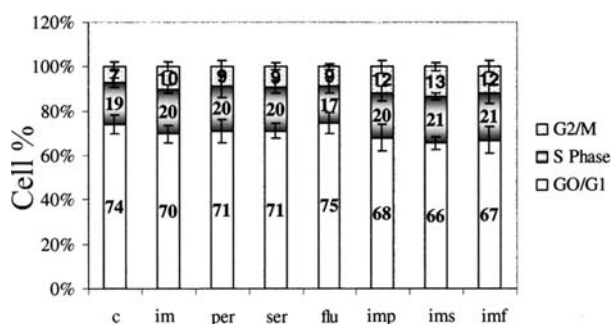


Figure 6. The effect of imatinib treatment combined with psychotropic drugs on cell cycle. U87 cells (3x10^{5}/10 ml) were treated with imatinib combined with perphenazine or sertraline or fluoxetine for 5 days. Nuclei were isolated, stained and DNA content was analyzed by FACS as described in Materials and methods. Results are expressed as mean ± SEM of at least 5 independent experiments performed in duplicates. c, control; im, imatinib (10$\mu$M); per, perphenazine (5 $\mu$M); ser, sertraline (7.5 $\mu$M); flu, fluoxetine (15 $\mu$M); imp, imatinib (10 $\mu$M) + perphenazine (5 $\mu$M); ims, imatinib (10 $\mu$M) + sertraline (7.5 $\mu$M); imf, imatinib (10 $\mu$M) + fluoxetine (15 $\mu$M). In ANOVA test-$F_{(7,78)}=10.799$, a small but significant elevation was found in G2/M: c vs. imp (p=0.007), c vs. ims (p<0.001), c vs. imf (p=0.001), ser vs. ims (p<0.001), im vs. ims (p=0.035), flu vs. imf (p=0.014).

or whether imatinib was added one day before, or one day after the addition of the psychotropic drugs (data not shown).

*The effect of combined treatment of imatinib with psychotropic drugs on cell cycle.* Since the combined treatment of imatinib and psychotropic drugs was the most efficient in their growth inhibitory effect, these combinations were further explored. To determine whether the synergistic antiproliferative effect of any combination was mediated via cell cycle arrest, we compared the cell cycle profile of the untreated U87 cells to that of the U87 cells following exposure for 2 and 5 days to 10 $\mu$M imatinib with or without 5 $\mu$M perphenazine, 7.5 $\mu$M sertraline or 15 $\mu$M fluoxetine. After 2 days of exposure no significant changes in cell cycle status were observed, except for a modest decrease in G0/G1 of cells treated with imatinib

(data not shown). After 5 days of combined treatment of cells, there were small but significant increases in G2/M, accompanied by a slight decrease in G0/G1. In contrast, no significant changes in the cell cycle profile were detected following the exposure to imatinib, or to each one of the three psychotropic drugs alone (Fig. 6).

These minor changes in the cell cycle profile could not explain the marked reduction in cell content after the exposure to the combined treatments. To investigate whether the synergistic decrease in cell content of the combined treatment of imatinib and psychotropic drugs is due to an apoptotic effect, we measured the pre-G1 phase of the cell cycle (analyzed by FACS) after 2 and 5 days of treatment. As shown in Table I, there were no significant increases in the pre-G1 fraction of each treatment alone after 2 or 5 days of exposure to the single drugs, compared to untreated cells. Only the combination of imatinib with fluoxetine resulted in a significant increase in the sub-G1 fraction following 5 days of combined treatment (Table I).

Similar results were obtained when DNA fragmentation was analyzed on day 5 using the cell death detection ELISA[Plus] kit. The apoptotic index increased significantly (43±7.6-fold, p<0.0001 vs. untreated cells) only in cells exposed to the combined treatment of imatinib 10 $\mu$M + fluoxetine 15 $\mu$M. In order to examine whether the increase in the apoptotic index following the combined treatment of imatinib with fluoxetine occurred via caspase-3 activation, we measured the specific activity of the enzyme 4, 8, 12 and 24 h after the addition of the drugs alone or in combination. Caspase 3 activity was not significantly affected by any type of treatment at all time point examined (data not shown).

*The effect of imatinib treatment combined with psychotropic drugs on the intracellular ATP content.* Previous studies from our and other laboratories revealed a reduction in intracellular ATP content following the treatment of cancer cells with psychotropic drugs (17,30). Imatinib was reported to reduce ATP content in cardiomyocytes (31). To examine whether the cell death of U87 cells occurred via an early
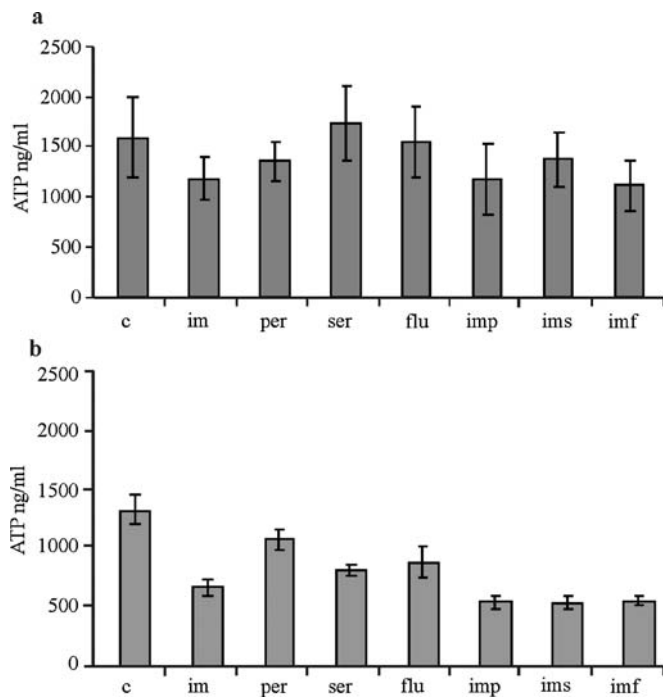
a



b



Figure 7. The effect of imatinib treatment combined with psychotropic drugs for 4 and 24 h on the intracellular ATP content. U87 cells (5x10⁵/10 ml) were incubated without control (c), or with imatinib (im; 10 μM), or psychotropic drugs [per, perphenazine (5 μM); ser, sertraline (7.5 μM); flu, fluoxetine (15 μM)] alone or in combination [ims, imatinib (10 μM) + perphenazine (5 μM); ims, imatinib (10 μM) + sertraline (7.5 μM); imf, imatinib (10 μM) + fluoxetine (15 μM)] for (a) 4 h or (b) 24 h. The results are expressed as means ± SEM of 3 independent experiments performed in duplicates. In the ANOVA test after 4 h (a): F(7,39)=0.535 c vs. im (p=1), c vs. per (p=1), c vs. ser (p=1), c vs. flu (p=1), c vs. imp (p=1), c vs. ims (p=1), c vs. imf (p=1). In the ANOVA test after 24 h (b): F(7, 50)=13.76 c vs. im (p=0.016), c vs. per (p=979), c vs ser (p=0.08), c vs. flu (p=0.694), c vs. imp (p=0.002), c vs. ims (p=0.002), c vs. imf (p=0.004).
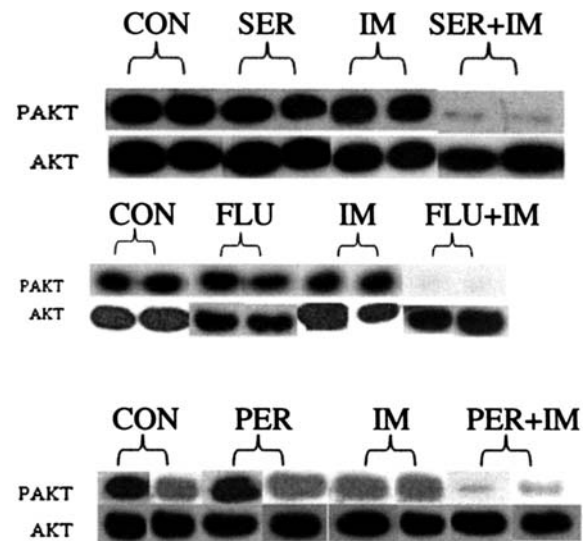


Figure 8. The effect of psychotropic drugs, imatinib and their combinations on the phosphorylation of AKT in U87 cells. U87 cells (5x10⁵/10 ml) were incubated without CON (control) or with IM (imatinib; 10 μM) or with psychotropic drugs [SER, sertraline (7.5 μM)] FLU, fluoxetine (15 μM), PER, perphenazine (5 μM)] alone or in combinations [SER + IM, sertraline (7.5 μM) + imatinib (10 μM); FLU + IM, fluoxetine (15 μM) + imatinib (10 μM); PER + IM, perphenazine (5 μM) + imatinib (10 μM)] for 24 h. The figure shows a representative experiment (one out of at least 6 consistent independent replications).
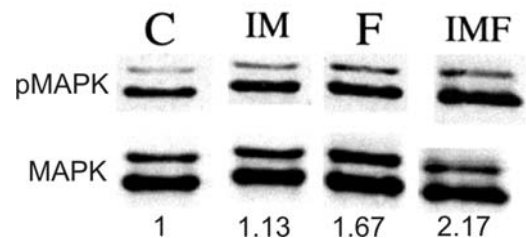


Figure 9. The effect of combined treatments of imatinib with fluoxetine on the phosphorylation of MAPK in U87 cells. U87 cells (1x10⁶/10 ml) were incubated without (C, control) or with imatinib (IM; 10 μM), fluoxetine (F; 15 μM) alone or in combination [IMF, imatinib (10 μM) + fluoxetine (15 μM)] for 24 h. The figure shows a representative experiment (one out of at least 3 consistent independent replications).

mitochondrial injury, we measured the intercellular ATP levels following single and combined exposure to imatinib and the psychotropic agents.

U87 cells were exposed to 10 μM imatinib or 5 μM perphenazine or 7.5 μM sertraline or 15 μM fluoxetine alone, or a combination of each of the psychotropic agents with imatinib for 4 and 24 h. No significant decrease in ATP level was observed in cells treated with 10 μM of imatinib for 4 h (Fig. 7a. ANOVA: F=0.535, DF=7, 39 NS). After 24 h of treatment, a marked significant reduction in ATP content was noted in cells treated with imatinib (Fig 7b. ANOVA: F=13.76, DF=7, 50, p<0.01). The psychotropic drugs alone did not significantly affect the intracellular ATP content. The combinations of imatinib with each one of the psychotropic agents resulted in a marked decrease in ATP content. However, this increase did not exceed that induced by the imatinib alone (Fig. 7a and b).

*The effect of imatinib treatment combined with psychotropic drugs on pAKT/AKT expression.* U87 cells highly express the phosphorylated form of AKT. Treatment of the cells for 24 h with 10 μM imatinib, 5 μM perphenazine, 7.5 μM sertraline, 15 μM fluoxetine did not affect the phosphorylation status of

AKT. However, treatment with imatinib combined with fluoxetine or sertraline resulted in a marked significant decrease (63%, 70% respectively, p<0.05 vs. control for both) in the phosphorylated form of AKT (F=6.08, DF=7.45, p<0.0001). The combination of perphenazine with STI 571 resulted in a mean 53% reduction in pAKT. A marked reduction was observed in 4 out of 6 independent experiments. However, the mean suppression did not reach statistical significance, p=0.2). The total AKT and ß-actin remained almost unchanged after all treatments administered (Fig. 8).

*The effect of imatinib treatment combined with psychotropic drugs on MAPK/pMAPK expression.* Treatment of U87 cells for 24 h with 10 μM imatinib or 15 μM fluoxetine led to a minor non-significant increase in the amount of the phosphorylated form of MAPK (pMAPK). The combined

treatment of imatinib with fluoxetine resulted in an increase (197%, p<0.01) in pMAPK expression. (F=6.84, DF=3.12, p<0.01). Total MAPK did not change significantly (Fig. 9). The effect of 5 $\mu$M perphenazine alone or in combination with 10 $\mu$M imatinib on pMAPK was not significant (data not shown).

## Discussion

In this study we demonstrated a synergistic anti-proliferative effect of the targeted therapeutic agent imatinib with the psychotropic agents, fluoxetine, sertraline (SSRIs) and perphenazine (a phenothiazine) on the U87 human glioblastoma cell line. We have previously reported similar synergistic interactions of imatinib with the psychotropic drug haloperidol (a sigma receptor ligand) in SK-MEL-28 human melanoma cells (17). The combination of imatinib with temozolomide, the currently most effective chemotherapeutic agent for the treatment of glioblastoma, resulted only in an additive anti-proliferative effect (Fig. 4). It is of note that pretreatment of a human glioblastoma cell line, RuSi RS1, with imatinib, significantly enhanced the cytotoxic effect of ionizing radiation (11). ABL kinase activity is implicated in DNA repair (32), and as an inhibitor imatinib can therefore potentiate DNA-damage caused by radiation or chemotherapeutic agents acting via DNA damage.

The combination of temolozomide with the psychotropic agents resulted in an additive anti-proliferative effect (Fig. 3), while the combination of irradiation and the psychotropic agents resulted in a less than additive effect on cell proliferation (Fig. 2). A recent report of Ren *et al* (33), showed a differential sensitivity of several glioblastoma cell lines to imatinib. Synergistic anti-tumor effects were found with the chemotherapeutic drugs temolozomide or hydroxyurea. However, in accordance with our results, no synergism could be detected on U87 cells. In this study we demonstrated for the first time a marked synergistic anti-proliferative effect of imatinib and psychotropic drugs on U87 cells (Fig. 5).

The cell cycle profile was not affected significantly by any of the single or combined treatments. Imatinib was shown to induce cell cycle arrest of glioma cells with a corresponding elevation in cyclin-dependent kinases. However these effects were not obtained in the U87 glioma cell line (33). The synergistic interaction between imatinib and the psychotropic agents on cell content does not seem to be related to a pro-apoptotic mechanism, since only one of the combinations (fluoxetine and imatinib) demonstrated an increase in sub-G1 fraction (Table I), which can also indicate other types of cell death. Moreover, the combined treatment did not alter caspase-3 activity, which is a marker of apoptosis. Interestingly, imatinib led to a significant reduction in intracellular ATP levels, which was not further augmented significantly by the addition of the psychotropic agents (Fig. 7). It may be speculated that the reduction of ATP induced by imatinib may contribute to a perturbation of the cell membrane, thus enhancing the effects of psychotropic agents, which have recently been reported to affect membranal lipid homeostasis (34).

In this study, we show a marked synergistic reduction in the expression of the key regulatory molecule pAKT, following the combined treatment of the cells with imatinib and the psychotropics, sertraline, perphenazine and fluoxetine (Fig. 8). This finding suggests that the anti-proliferative synergism may be mediated by the marked decrease in pAKT levels. It should be noted that in our study, imatinib alone did not alter pAKT cellular expression. Other studies have shown different effects on pAKT, depending on the cell line used (19).

Since MAPK is a major regulator of cell proliferation, we assessed the effects of imatinib and the psychotropic drugs alone and in combination, on its expression. None of the single or combined treatments led to a reduction in the expression of the active phosphorylated form of MAPK (pMAPK). However, the combination of fluoxetine with imatinib resulted in an increase of pMAPK (Fig. 9). The role of this activation in the synergistic anti-proliferative effect of fluoxetine and imatinib in U87 cells is currently unclear. It is of interest to note that the activation of MAPK by sertraline in hepatoma cells and fluoxetine in rat astrocytes has previously been reported (22,35). It should also be noted that in several osteosarcoma cell lines, imatinib was shown to reduce pAKT, while pMAPK remained constitutively activated (36). In a recent study it has been reported that the flavonoid, n arginine, inhibited vascular smooth muscle proliferation via the activation of the ERK signaling pathway (37).

It is known that imatinib uptake into cells is often mediated by the OCT1 transporter, with MDR-1 being responsible for efflux (38-40). It is therefore possible that the effects observed in this study relate to effects on intracellular concentrations of imatinib. Although the concentrations of the psychotropic agents used in this and other *in vitro* studies were beyond the clinically relevant blood levels achieved in humans treated for psychiatric disorders, recent studies have demonstrated an anti-proliferative effect of sertraline in an *in vivo* human colon cancer model (41). Thus, it seems that further *in vivo* studies combining imatinib with psychotropic agents, especially fluoxetine and sertraline, are warranted.

## Acknowledgements

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