

## COMBINED ANTIPROLIFERATIVE ACTIVITY OF IMATINIB MESYLATE (STI-571) WITH RADIATION OR CISPLATIN *IN VITRO*

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Little is known about the interaction of novel anticancer drugs with other treatment modalities. *The aim* of this study was to examine the effect of combining imatinib mesylate (STI-571) with radiation or cisplatin on the survival of two human solid tumor cell lines – SKNMC cells derived from Ewing sarcoma and breast cancer MCF-7 cells. *Methods:* Cell proliferation was determined using the sulphorodamine B cytotoxicity assay. Cell cycle analysis was performed with flow cytometry. Apoptosis was determined using a commercial cell death ELISA plus kit. Phosphorylated AKT, which has been suggested to be involved in radiation resistance, was detected by Western blot analysis. *Results:* Exposure of SKNMC cells to STI-571 resulted in a dose-dependent antiproliferative effect and a decrease in phosphorylated AKT expression. There was no evidence of apoptosis. The combination of STI-571 with radiation or cisplatin had an additive antiproliferative effect in SKNMC cells (60% reduction in cell number). A similar effect was observed in human MCF-7 breast cancer cells. *Conclusion:* STI-571 improves the outcome of cisplatin or irradiation treatment *in vitro*. AKT pathway may play a role in the additive effect of STI-571 and irradiation.

**Key Words:** STI-571, gleevec, imatinib mesylate, irradiation, cisplatin, cell cycle, phosphorylated AKT, apoptosis.

More than 50% of patients with cancer require radiation as adjuvant or palliative treatment. Combining radiation with cytotoxic chemotherapeutic agents has become common practice. However, little is known about the interactions of the novel antitumor drugs, such as STI-571, with standard treatment [1–4].

STI-571 (imatinib mesylate; Gleevec) has been found to be effective in the treatment of chronic myeloid leukemia (CML) and gastrointestinal stromal tumors [5–9]. In CML, it exerts its antitumor action by inhibiting the phosphorylation of downstream proteins involved in BCR-ABL signal transduction. STI-571 also affects receptor tyrosine kinases, namely c-kit and platelet-derived growth factor (PDGF) receptors [10].

The effect of the interaction of STI-571 and radiation on Abl-expressing cells remains unclear. One study found that fibroblasts from Abl knockout mice have reduced sensitivity to ionizing radiation [11], whereas later reports contradicted this result [2]. To explain the possible interaction between radiation and STI-571, some authors suggested that STI-571 may be involved in the inhibition of Rad 51 expression. Rad 51 is a component of the DNA repair pathway, and its reduction would be expected to enhance radiation sensitivity [12, 13]. However, although STI-571 re-

duced Rad 51 levels in glioma cells, it was unable to completely eliminate the radiation-induced increase in Rad 51, suggesting the presence of an additional signaling process [3].

The activation of the phosphatidylinositol 3 kinase (PI3K)/AKT signal transduction pathway may be a major contributor to radioresistance as well as to cisplatin resistance [14, 15]. AKT, the key protein in this pathway, was reported to be involved in enhancing cell proliferation and inhibiting apoptosis [16]. Thus, the (PI3K)/AKT pathway is a promising target for novel anticancer agents.

In the present study, we examined the effect of combined treatment with STI-571 and radiation or cisplatin on SKNMC, a cell line derived from Ewing sarcoma, and on the human MCF-7 breast cancer cell line. SKNMC is characterized by overexpression of the c-kit receptor [17], whereas MCF-7 cells exhibit low c-kit-receptor expression. Both cell lines display radiation sensitivity.

### MATERIALS AND METHODS

STI-571 was kindly donated by Novartis Pharmaceutical Inc. (Basel, Switzerland). Sulforhodamine B (SRB) was obtained from Sigma (St. Louis, MO). RPMI 1640, fetal calf serum (FCS) and antibiotics (penicillin and streptomycin) were purchased from Biological Industries (Beth Ha'Emek, Israel). The chemotherapeutic agent cisplatin (Abiplatin) was obtained from ABIC (Netanya, Israel).

**Cell cultures.** The SKNMC cell line, derived from peripheral primitive neuroectodermal tumor, was

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**Abbreviations used:** CML – chronic myeloid leukemia; PDGF – platelet-derived growth factor; PI3K – phosphatidylinositol 3 kinase; STI-571 – imatinib mesylate.

kindly donated by Dr. Gad Lavie, Sheba Medical Center, Israel. MCF-7, a human breast cancer cell line, was purchased from ATCC. Both cell lines were cultured in RPMI 1640 supplemented with 10% FCS and antibiotics (penicillin, streptomycin). The cells were incubated at 37 °C in a 5% CO<sub>2</sub>, 95% humidified atmosphere.

Incubation of the SKNMC or MCF-7 cells in the presence of increasing concentrations of STI-571 (0, 5, 10, 15, 20 μM) for 5 days was performed to determine the concentration of STI-571 required to cause a 50% decrease in cell number (IC<sub>50</sub>).

**Cytotoxicity assay.** Cells (1.5 × 10<sup>4</sup>/ml) were seeded in quadruplicate in 24-well plates with increasing concentrations (0, 5, 10 μM) of STI-571 and cultured for 5 days. Twenty four hours after seeding, the cells were irradiated at increasing doses (0, 200, 400, 600 cGY) with a 6MV linear accelerator (Varian 600C, Palo Alto, CA, USA). Five days after seeding, cytotoxicity was determined with the SRB assay [18].

To test the combined effect of STI-571 and cisplatin, the two agents were added to the culture medium together for 5 days. STI-571 (5 or 10 μM) was added to 0.05 and 0.1 μg/ml cisplatin for experiments on the SKNMC line, and to 0.05 and 0.25 μg/ml cisplatin for experiments on the MCF-7 line. Cytotoxicity was tested on day 5 with the SRB assay.

**SRB staining.** In brief, the medium was removed, and cold 10% trichloroacetic acid (TCA) was added for 1 h at 4 °C. The TCA was then removed, and the plates were rinsed with water and stained with SRB, 4 mg/ml in 1% acetic acid, as described [12]. The bound SRB was solubilized in 1 ml of 10 mM unbuffered Tris solution. Thereafter, 100 μl of each sample were transferred to a 96-well plate and read at 550 nm with a microtiter ELISA reader. The results were expressed as percentage of the control.

**Calculation.** The inhibitory effect of each agent added to the cultures was calculated as follows: Inhibition (%) = [1-(SRB staining in treated wells/SRB staining in control wells)] X100.

The theoretical additive inhibitory effect of the agents *a* and *b* was calculated using the following equation:  $lab = 100 \times [1 - (1 - la/100) \times (1 - b/100)]$  where *lab* is the calculated additive inhibitory effect expressed as % inhibition. *la* and *lb* are the measured inhibitory effects (%) of each agent acting alone as compared with that of the control cultures. This equation was derived assuming the inhibitory agents act independently on the same target population [19].

**Western blot.** Phosphorylated AKT was detected in the cells after induction with FCS as follows. Cells (1 × 10<sup>6</sup>/dish) were grown in serum-deprived RPMI 1640 for 24 h. STI-571 was then added to the culture for 90 min. To induce phosphorylation of AKT, FCS was added to the cell cultures for 1 h. Cells were then harvested, washed with PBS and lysed with the CHAPS lysis buffer (10 mM Tris-HCl, pH 7.5, 1 mM MgCl<sub>2</sub>, 1 mM EGTA, 0.1 mM benzamidine, 5mM β-mercaptoethanol, 0.5% 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate, 10% glycerol). Protein concentration was determined

using the Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA). Equal protein amounts of all samples (20–40 μg) were resolved on 10% sodium dodecylsulfate (SDS) and transferred to a polyacrylamide gel and then to a polyvinylidene difluoride (PVDF) membrane. The AKT protein or its phosphorylated form was detected with a specific monoclonal antibody (Cell Signaling Technology, Beverly, MA) in 1 : 1000 dilution, followed by horseradish peroxidase-conjugated goat-anti-rabbit antibody (Jackson Laboratories, West Grove, PA, USA). The SuperSignal<sup>®</sup>West Pico Chemiluminiscent Substrate kit (Pierce, IL, USA) was used to visualize the expression of both proteins, according to the manufacturer's protocol. Signals were quantified using Quantity-One software for Bio-Rad image analysis systems (Bio-Rad Laboratories). Phosphorylated AKT expression was calculated relative to the total signal obtained from the AKT protein.

**Apoptosis assay.** Cells (1.5 × 10<sup>4</sup>/ml) were seeded in a 24-well plate and treated with STI-571 10 or 15 μM, cisplatin 0.1 μg/ml, and radiation 400 cGY, alone or in combination. DNA fragmentation was determined by nucleosome assessment using a commercial Cell Death ELISA Plus kit (Roche, Mannheim, Germany), according to the manufacturer's instructions. Cell content was estimated in identical plates using the SRB method. The data obtained by ELISA were normalized for cell content; the apoptotic index was calculated as percentage of the untreated controls.

**Statistical analysis.** The data are presented as mean ± SD. Each experiment was performed at least three times. The data were analyzed with two-way analysis of variance (ANOVA) using SPSS software. A *p* value of less than 0.05 was considered statistically significant.

## RESULTS

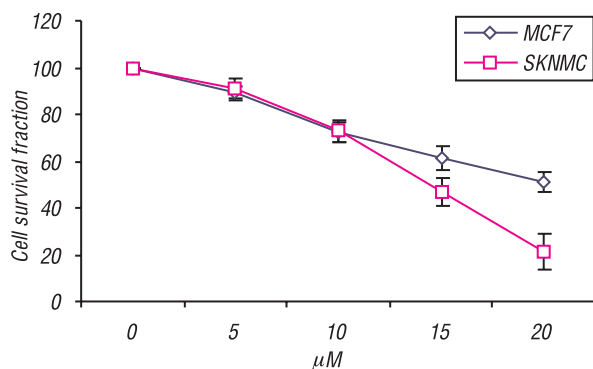
**Effect of STI-571 on cell proliferation and apoptosis.** Incubation of the SKNMC or MCF-7 cells in the presence of increasing concentrations of STI-571 for 5 days resulted in a concentration-dependent decrease in cell number.

The concentration of STI-571 required to cause a 50% decrease in cell number (IC<sub>50</sub>) was about 15 μM for the SKNMC line and 20 μM for the MCF7 line. As the dose increased, the sensitivity of the SKNMC cells to STI-571 increased compared to the sensitivity of the MCF-7 cells (Fig. 1). Therefore, further evaluation of apoptosis and analysis of the cell cycle were performed on SKNMC cells only.

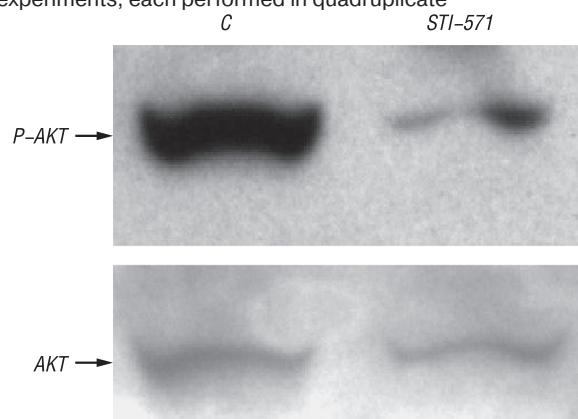
The data in the Table show that STI-571 had only a negligible effect on the apoptotic index.

**Effect of STI-571 on phosphorylated AKT levels.** The levels of AKT protein previously reported to be involved in enhancing cell proliferation, inhibiting apoptosis, and contributing to radioresistance were evaluated by Western blot analysis.

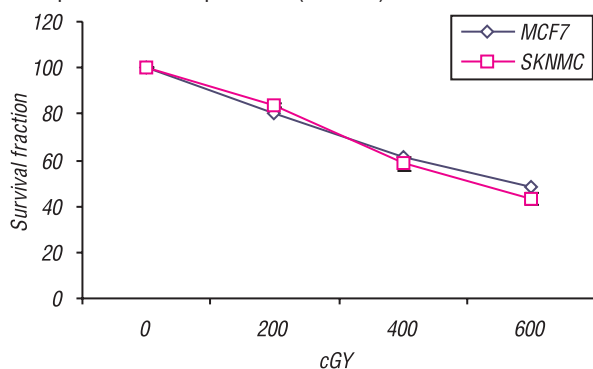
As shown in Fig. 2, phosphorylated AKT decreased dramatically by 72% after 90 min exposure of SKNMC to 15 μM STI-571, as it has recently been reported by us, while exploring regulation of telomerase activity by STI-571 [20].



**Fig. 1.** Effect of STI-571 on SKNMC and MCF-7 Cells. SKNMC and MCF-7 cells ( $1.5 \times 10^4$ /ml) were incubated in the presence of increasing concentrations of STI-571, as described in Material and Methods. The surviving fraction of SKNMC and MCF-7 cell lines was determined with the sulforhodamine-B staining method. Values represent mean  $\pm$  SD of three independent experiments, each performed in quadruplicate



**Fig. 2.** Phosphorylated AKT expression in response to STI-571 treatment. Phosphorylated AKT and total AKT protein levels after SKNMC exposure to 15  $\mu$ M STI-571 were analyzed by Western blot, as described in Material and Methods. The figure depicts one representative experiment (out of 3)



**Fig. 3.** Effect of radiation on SKNMC and MCF-7 Cells. SKNMC and MCF-7 cells ( $1.5 \times 10^4$ /ml) were incubated in the presence of increasing concentrations of STI-571, as described in Material and Methods. The surviving fraction of SKNMC and MCF-7 cell lines was determined by SRB staining. Values represent mean  $\pm$  SD of three independent experiments, each performed in quadruplicate

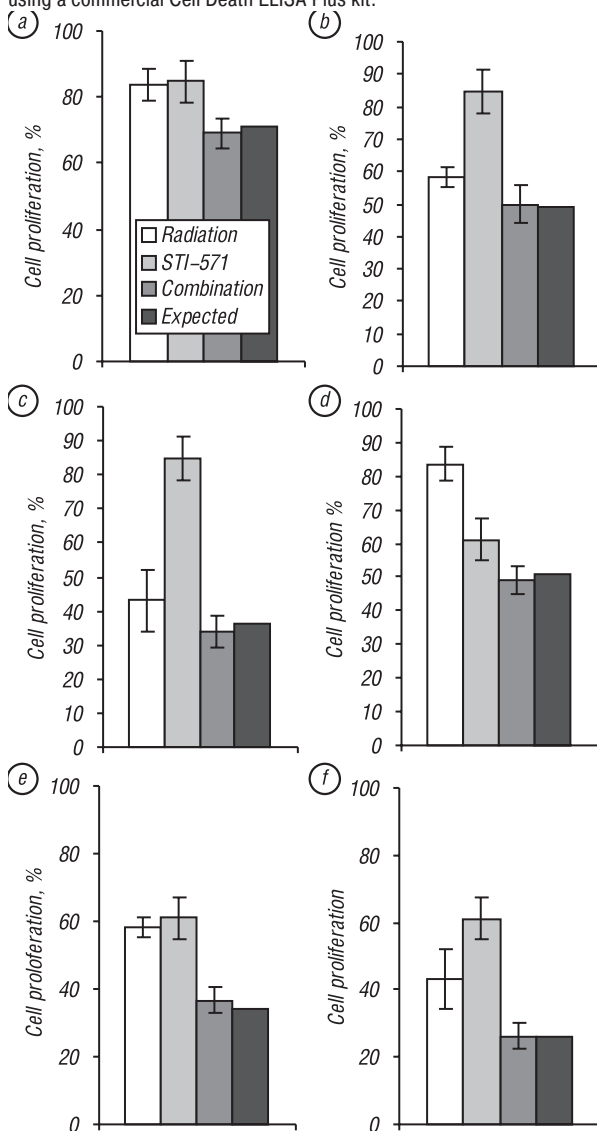
**Exposure of SKNMC and MCF-7 cells to combination of STI-571 and radiation.** Fig. 3 shows the effect of radiation alone on SKNMC and MCF-7 cancer cells, and Fig. 4 and 5 show the effect of the combination of STI-571 and radiation. Combined treatment resulted in an additive decrease in cell number, confirmed by two-way ANOVA. This effect was more

pronounced in the SKNMC than the MCF-7 cells. Radiation induced a marked increase in the apoptotic index of SKNMC cells. The addition of STI-571 did not augment the apoptotic effect of radiation (Table).

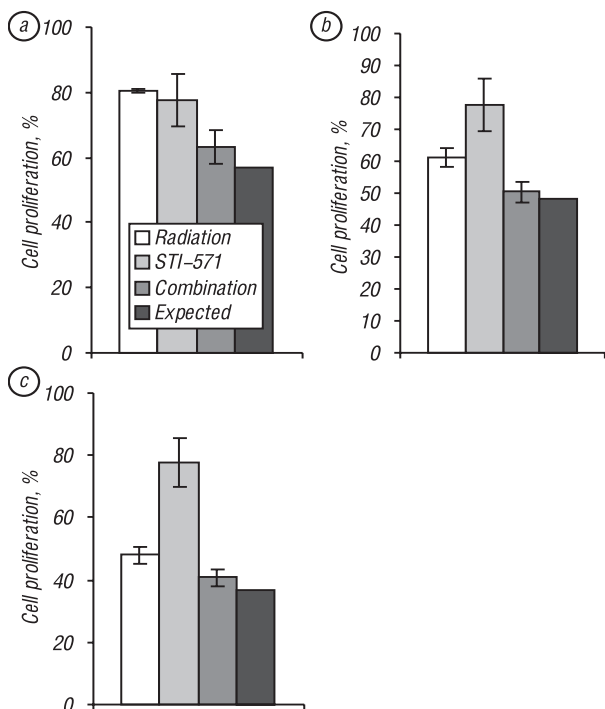
**Table.** Effect of STI-571, cisplatin, radiation and their combination on the apoptotic index of SKNMC cells\*

Modalities alone or combined*	Apoptotic index (%)
Control	1
STI-571 10 $\mu$ M	1.19
STI-571 15 $\mu$ M	1.28
Cisplatin 0.1 $\mu$ g/ml	1.27
STI 10 $\mu$ M cisplatin 0.1 $\mu$ g/ml	1.78
400 cGY	4.1
STI 10 $\mu$ M + 400 cGY	3.99

\*Cells were treated with STI-571 10 and 15  $\mu$ M, cisplatin 0.1  $\mu$ g/ml and irradiation of 400 cGY, alone or in combination. Cells were seeded in a 24-well plate, and DNA fragmentation was determined by nucleosome assessment using a commercial Cell Death ELISA Plus kit.



**Fig. 4.** Combined effect of STI-571 and radiation on SKNMC cell line. Cells ( $1.5 \times 10^4$ /ml) were incubated with 5 and 10  $\mu$ M STI-571. Twenty-four hours after seeding, the treated and untreated cells were irradiated at increasing doses (0, 200, 400, 600 cGY) with a 6MV linear accelerator. The surviving fraction of SKNMC cell lines was determined by SRB staining. Values represent mean  $\pm$  SD of three independent experiments, each performed in quadruplicate. (a) 5  $\mu$ M STI-571 and 200 cGY; (b) 5  $\mu$ M STI-571 and 400 cGY; (c) 5  $\mu$ M STI-571 and 600 cGY; (d) 10  $\mu$ M STI-571 and 200 cGY; (e) 10  $\mu$ M STI-571 and 400 cGY; (f) 10  $\mu$ M STI-571 and 600 cGY



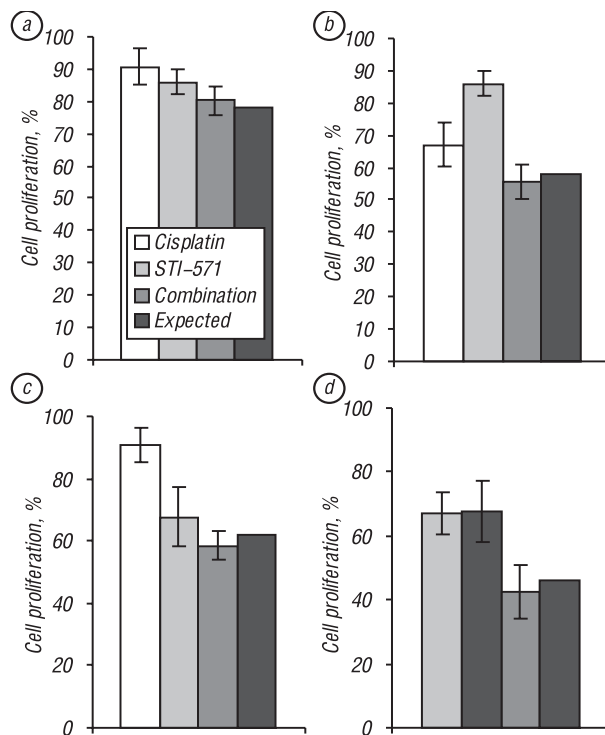
**Fig. 5.** Combined effect of STI-571 and radiation on MCF-7 cell line. Cells ( $1.5 \times 10^4$ /ml) were incubated with 10 μM STI-571. Twenty-four hours after seeding, the treated and untreated cells were irradiated at increasing doses (0, 200, 400, 600 cGY) with a 6MV linear accelerator. The surviving fraction of SKNMC and MCF-7 cell lines was determined by SRB staining. Values represent mean  $\pm$  SD of three independent experiments, each performed in quadruplicate. (a) 10 μM STI-571 and 200 cGY; (b) 10 μM STI-571 and 400 cGY; (c) 10 μM STI-571 and 600 cGY

**Exposure of SKNMC and MCF-7 cells to STI-571 and cisplatin.** Incubation of the SKNMC or MCF-7 cells in the presence of increasing concentrations of cisplatin resulted in a concentration-dependent decrease in cell survival. The concentration of cisplatin required to inhibit 50% cell growth (IC50) was found to be around 0.35 μg/ml for the MCF-7 cell line and 0.09 μg/ml for the SKNMC cell line.

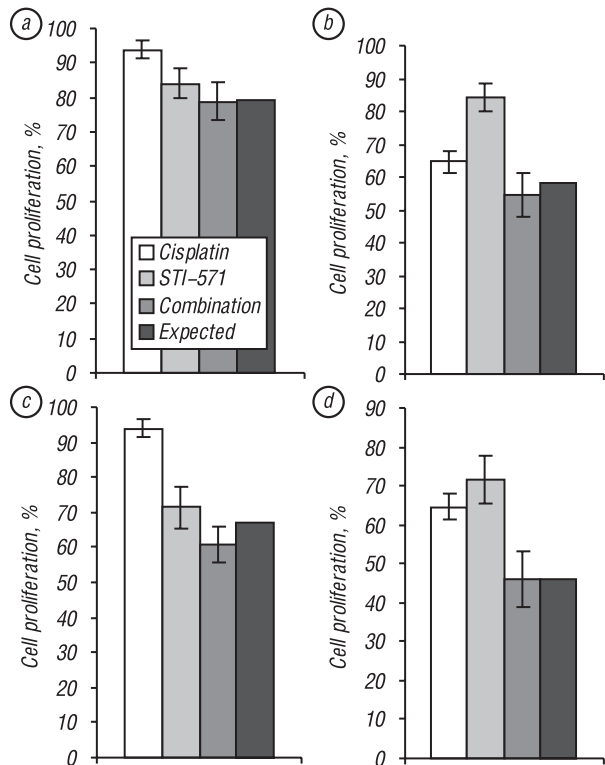
Incubation of the SKNMC and MCF-7 cells in the presence of combined STI-571 and cisplatin for 5 days yielded an additive cell-killing effect on both cell lines (Fig. 6, 7), with only a slight increase in apoptosis compared to each agent alone (Table).

**DISCUSSION**

To improve the outcome of radiation treatment, clinicians combine its use with standard cytotoxic chemotherapeutic agents. Since the introduction of novel anticancer drugs to the daily treatment armamentarium, researchers have been seeking data on the potential benefits of their interaction with standard treatment modalities. The present study shows that STI-571 and radiation have an additive antiproliferative effect on SKNMC and MCF-7 human solid tumor cell lines. The results are in accordance with the report of Topaly et al. [4] who found a synergistic effect of STI-571 and radiation in BCR-Abl-positive lymphoid and myeloid blast crisis cells. However the Abl family proteins are non receptor tyrosine kinase and different intracellular mechanisms may be involved.



**Fig. 6.** Combined effect of STI-571 and cisplatin on SKNMC cell line. STI-571 at concentrations of 5 and 10 μM was added to 0.05 and 0.1 μg/ml cisplatin. (a) 5 μM STI-571 and 0.05 μg/ml cisplatin; (b) 5 μM STI-571 and 0.1 μg/ml cisplatin; (c) 10 μM STI-571 and 0.05 μg/ml cisplatin; (d) 10 μM STI-571 and 0.1 μg/ml cisplatin. The surviving fraction was determined by SRB staining. Values represent mean  $\pm$  SD of five independent experiments



**Fig. 7.** Combined effect of STI-571 and cisplatin on MCF-7 cell line. STI-571 at concentrations of 5 and 10 μM was added to 0.05 and 0.25 μg/ml cisplatin. (a) 5 μM STI-571 and 0.05 μg/ml cisplatin; (b) 5 μM STI-571 and 0.25 μg/ml cisplatin; (c) 10 μM STI-571 and 0.05 μg/ml cisplatin; (d) 10 μM STI-571 and 0.25 μg/ml cisplatin. The surviving fraction was determined by SRB staining. Values represent mean  $\pm$  SD of five independent experiments



Since AKT plays a major role in the PI3 kinase signal transduction pathway, and also largely contributes to radioresistance, we sought to determine the effect of combined treatment on levels of phosphorylated AKT (the active form of AKT). We noted a marked decrease in phosphorylated AKT following STI-571 treatment of SKNMC cells. Our data are in accordance with the report of Ohashi et al. [21] who found a reduction in phosphorylated AKT and its downstream targets in cells expressing mutant platelet-derived growth factor receptor- $\alpha$ .

We speculate that the inhibition of these pathways may have contributed to the additive antiproliferative effect of STI-571 and radiation. In light of findings that p-AKT inhibits apoptosis, we checked apoptosis in the SKNMC cell line; however, no significant contribution of the drug to apoptotic death was detected. These results suggest that STI-571 is cytostatic rather than cytotoxic to SKNMC cells, and join previous findings of a possible cytostatic character of STI-571 [22, 23].

The cell response to irradiation is affected by the cell cycle phase. The mitotic phase is the most sensitive, followed by the G<sub>2</sub> phase. Resistance to radiation gradually increases as the cells proceed through the late G<sub>1</sub> and S phases, reaching a maximum in the late S phase. In cells with a long G<sub>1</sub> phase, a peak of resistance is seen early in G<sub>1</sub> [24, 25]. Our earlier study showed that 4 days of treatment of cells with 15  $\mu$ M STI-571 resulted in a fivefold increase in the percent of cells in G<sub>2</sub>/M phase (15% vs 3.26%,  $p = 0.0013$ ). This increase was accompanied by a concomitant decrease in cells in the S phase, from 36.5% to 26.9% ( $p = 0.0029$ ) [20].

On the basis of these data, we examined the influence of STI-571 on the cell cycle. Our study revealed that within 24 h of incubation — the time at which we irradiated the cells — STI-571 had no influence on the cell cycle. There was, however, a small but significant increase in the G<sub>2</sub>/M phase 4 days after incubation with STI-571 [20]. Further evaluation of the effect of cell irradiation 4 days after incubation with STI-571 yielded no significant improvement in inhibition of cell growth as compared to radiation 24 h after incubation.

Cisplatin is a major drug in the treatment of malignancy. Our study showed that the addition of a novel drug to an “old” one augments cell death. Accordingly, it was reported that the combination of STI-571 and cisplatin synergistically inhibited lung [1] or head and neck [27, 28] cancer cell growth. Regardless of whether their action is additive or synergistic, STI-571 and cisplatin do not interfere with each other’s antiproliferative effect on breast cancer and Ewing’s sarcoma as was observed in our experiments.

These promising preliminary findings may have important implications for the treatment of various types of cancer and should support conducting clinical trials with this new agent [29]. Further studies are needed to corroborate the benefit of combining STI-571 with standard modalities.

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## АНТИПРОЛИФЕРАТИВНАЯ АКТИВНОСТЬ ИМАТИНИБА (STI-571) В КОМБИНАЦИИ С ОБЛУЧЕНИЕМ ИЛИ ЦИСПЛАТИНОЙ *IN VITRO*

**Цель:** оценить антипролиферативный эффект иматиниба (STI-571) в комбинации с облучением или цисплатиной по отношению к двум клеточным линиям – клеткам линии SKNMC, полученным из саркомы Эвинга, и клеткам рака молочной железы человека линии MCF-7. **Методы:** для оценки пролиферации клеток применяли метод анализа цитотоксичности с использованием сульфородамина В. Для анализа распределения клеток по фазам клеточного цикла применяли метод проточной цитометрии, апоптоза – с применением коммерческого набора для проведения ИФА. Уровень фосфорилированной киназы АКТ, предположительно связанной с радиорезистентностью, определяли методом Вестерн-блот анализа. **Результат:** инкубация клеток SKNMC с STI-571 приводила к дозозависимому антипролиферативному эффекту и снижению фосфорилирования АКТ, но не апоптозу клеток. Комбинированное применение STI-571 и облучения или цисплатины оказывало дополнительное антипролиферативное воздействие на клетки линии SKNMC (60% уменьшения количества клеток). Аналогичные эффекты отмечали на клетках линии MCF-7. **Выводы:** обработка опухолевых клеток STI-571 усиливает эффект облучения и цисплатины *in vitro*, причем таковой может быть опосредован сигнальным каскадом АКТ.

**Ключевые слова:** STI-571, глибек, иматиниб, облучение, цисплатина, клеточный цикл, фосфорилированная АКТ, апоптоз.