

## The effects of sunitinib malate used in targeted therapy on the proliferation of HeLa cells *in vitro*

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### Summary

**Purpose:** In this study, the antiproliferative and apoptotic effects of sunitinib (SU-11248, Sutent) which is used for targeted therapy was evaluated on HeLa cell line originated from human cervix carcinoma.

**Methods:** Three different doses of sutent ( $D_1 = 1 \mu\text{M}$ ,  $D_2 = 5 \mu\text{M}$ ,  $D_3 = 10 \mu\text{M}$ ) were administered to cells for 72 h to determine the optimal dose.

**Results:** Increase in apoptotic index (AI), decrease in mitotic index (MI) of cells and slow down in proliferation rate were achieved at the dose level of  $10 \mu\text{M}$ , especially at 72 h. All these findings were statistically significant ( $p < 0.001$ ). In addition, anaphase bridges and existence of tripolar metaphase cells that were observed at 72 h were possibly attributable to a chromosomal instability arising from shortening of telomere.

**Conclusion:** In this study, sutent effected cell kinetic parameters significantly. These results are consistent with other studies in the literature. In addition, anaphase bridges which were seen in mitosis preparations were interpreted as shortening or degradation of the telomere.

**Key words:** apoptotic index, cancer, mitotic index, sunitinib, sutent, targeted cancer therapy

## Introduction

Cancer is an abnormal growth of cells caused by multiple changes in gene expression leading to dysregulated balance of cell proliferation and cell death and ultimately evolving into a population of cells that can invade tissues and metastasize to distant sites, causing significant morbidity and, if untreated, death of the host [1]. Chemotherapy is one of the conventional methods used for the treatment of cancer. Conventional chemotherapy, although directed toward certain macromolecules or enzymes, typically does not discriminate effectively between rapidly dividing normal cells and tumor cells, thus leading to several toxic side effects [2]. Therefore in recent years targeted therapies have been started to develop. Targeted cancer therapies are drugs or other substances that block the growth and spread of cancer by interfering with specific molecules involved in tumor growth and progression [3]. One of the drugs used in targeted therapies is sunitinib (SU-11248, sunitinib). Sunitinib is a small-molecule receptor tyrosine kinase inhibitor that inhibits cellular signaling of multiple targets, approved for advanced renal cell carcinoma and imatinib-resistant or imatinib-intolerant gastrointestinal stromal tumors [4,5]. Sunitinib inhibits at least 8 receptor protein-tyrosine kinases including vascular endothelial growth factor receptors 1–3 (VEGFR1–VEGFR3), platelet-derived growth factor receptors (PDGFR $\alpha$  and PDGFR $\beta$ ), stem cell factor receptor (Kit), Flt-3, and colony-stimulating factor-1 receptor (CSF-1R) [6].

In this study we investigated the antiproliferative and apoptotic effects of sunitinib on HeLa cell line originated from human cervix carcinoma. Three different doses of sunitinib were administered to the cells in order to determine the optimal dose from 12 to 72 h. The effects of sunitinib on the growth rates of HeLa cells were evaluated with the WST-1 assay kit, while the MI and AI were determined with using light and fluorescence microscope, respectively.

## Methods

### Cell culture

The HeLa cell line used in this study was obtained from European Cell Culture Collection (ECACC). Cells were cultured in Medium-199 (M-199, Sigma, USA)

containing 10% fetal bovine serum (FBS, Gibco Lab), 100  $\mu$ g/ml streptomycin (Streptomycin sulphate, I. E. Ulugay), 100 IU/ml penicillin (Pronapen, Pfizer), amphotericin B (Sigma, USA) and 2 mM glutamine at 37 °C in humidified atmosphere of 5% CO<sub>2</sub>. The pH of the medium was adjusted to 7.4 with NaHCO<sub>3</sub>.

### Drug doses

Sunitinib concentrations that were used in the present study were determined based on previous *in vitro* and clinical studies. First, 1 mM stock solution was prepared with M-199 supplemented with 10% FBS. Three different doses were obtained by dilution of the stock solution and were determined as dose 1 (D<sub>1</sub>) = 1  $\mu$ M, dose 2 (D<sub>2</sub>) = 5  $\mu$ M and dose 3 (D<sub>3</sub>) = 10  $\mu$ M. HeLa cell cultures were exposed to the 3 doses for 12, 24, 48 and 72 h.

### Determination of cytotoxic activity with WST-1 (mitochondrial dehydrogenase enzyme activity)

The effects of sunitinib on the growth rates of HeLa cells were evaluated with the WST-1 assay kit (Roche). The WST-1 assay was applied to identify the cytotoxicity of sunitinib after 12, 24, 48 and 72 h of exposure. For WST-1 assay cells were cultured in 96-well plates in a final volume of 200  $\mu$ l/well culture medium in a humidified atmosphere. Twenty  $\mu$ l of the cell proliferation reagent WST-1 were added to each well. Cells were incubated for 4 h in a humidified atmosphere. At the end of this period, cells were shaken thoroughly for 1 min on the shaker. Then, absorbance of the samples was measured against a background control as a blank using a ELISA reader ( $\mu$ Quant, Bio-Tek Instruments Inc, USA) at 420–480 nm [7].

### Determination of optimal dose with mitochondrial dehydrogenase enzyme activity analysis

In this examination 3 different doses of sunitinib (D<sub>1</sub> = 1  $\mu$ M, D<sub>2</sub> = 5  $\mu$ M, D<sub>3</sub> = 10  $\mu$ M) were applied to HeLa cell culture for 72 h and the cytotoxic effects of these doses were evaluated with mitochondrial dehydrogenase enzyme activity analysis (WST-1 assay). As a result of this application, absorbance values were measured for each dose and the most effective dose for HeLa cell culture was determined.

### Mitotic index analysis

The MI was studied using the Feulgen method [8,9]. Before the cells were stained with Feulgen, they were prepared with 1 N HCl at room temperature for 1 min and then hydrolyzed with 1 N HCl for 10.5 min at 60°C. After slides were stained with Feulgen, they were rinsed for a few minutes in distilled water and stained with 10% Giemsa stain solution at pH 6.8 for 3 min and washed twice in phosphate buffer. After staining, the slides were rinsed in distilled water and then were air-dried. Finally, the MI was calculated by counting metaphases, anaphases and telophases for each control group which was not exposed to sunitinib and the experimental groups which were exposed to D<sub>3</sub> dose (10µM) of sunitinib for 12, 24, 48 and 72 h. At least 3,000 cells were examined using light microscope from each slide to determine the MI.

### Apoptotic index analysis

The AI (the percentage of cells undergoing apoptosis) was studied using fluorescence microscope. For the determination of the AI, cells were fixed with methanol and stained with 4'-6 diamidine-2 phenylindol

(DAPI). Following extensive washing in phosphate-buffered saline (PBS), slides were scored under fluorescence microscope. For evaluation of the AI at least 100 cells were counted for the control and each experimental group.

### Statistics

Values of proliferation rate, MI and AI were evaluated relative to controls and to each other. For this reason, values obtained from all experimental groups were analyzed using one-way ANOVA test. The significance between control and experimental groups was determined by DUNNETT's test and the significance between experimental groups was determined by Student's t-test.

## Results

**Determination of optimal dose with mitochondrial dehydrogenase enzyme activity analysis** The absorbance values of each dose for 72 h are shown in Table 1. All the differences between control and experimental groups were statistically significant ( $p < 0.001$ ). In addition, significant differences among the experimental groups were noted ( $p < 0.001$ ) (Figure 1). Seventy-two hours after drug exposure, mitochondrial dehydrogenase enzyme activity values were 66% for D<sub>1</sub>, 46% for D<sub>2</sub> and 23% for D<sub>3</sub> compared with the control group which was considered 100% (Figure 2).

### Determination of cytotoxic activity with WST-1 (mitochondrial dehydrogenase enzyme activity analysis)

Amongst the three different sunitinib doses administered to the cells for 72 h, D<sub>3</sub> dose of sunitinib that inhibited the cell proliferation effectively compared to the other

**Table 1.** Absorbance values of mitochondrial dehydrogenase enzyme activity of HeLa cells treated with 3 different doses of sunitinib (D<sub>1</sub> = 1µM, D<sub>2</sub> = 5µM, D<sub>3</sub> = 10µM) (±SD)

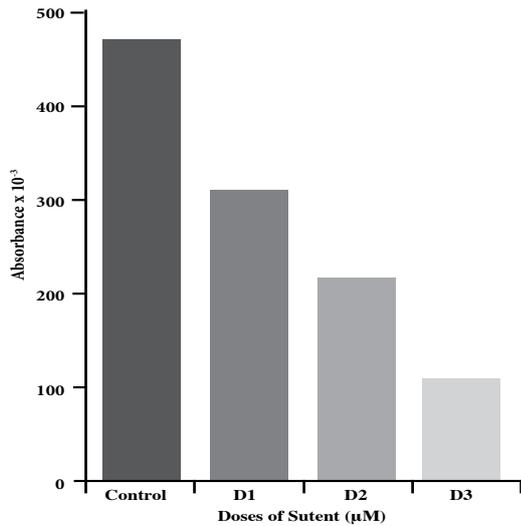
Groups	Absorbance values (450-690 nm)
Control	472.231x10 <sup>-3</sup> * ± 0.32
D <sub>1</sub>	312.231x10 <sup>-3</sup> ± 0.29
D <sub>2</sub>	217.423x10 <sup>-3</sup> ± 0.43
D <sub>3</sub>	111.625x10 <sup>-3</sup> ± 0.52

SD: standard deviation. \*Significantly different ( $p < 0.01$ )

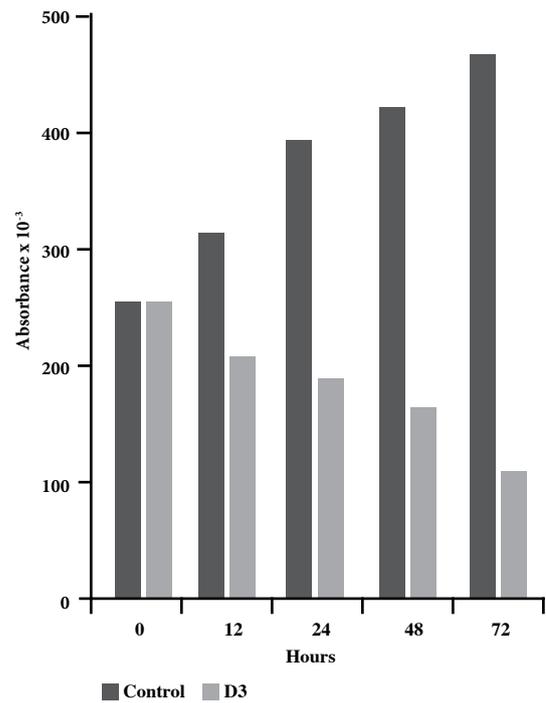
**Table 2.** Absorbance values of mitochondrial dehydrogenase enzyme activity of HeLa cells treated with D<sub>3</sub> dose of sunitinib for 0-72 h (±SD)

Experimental groups Time of exposure (hours)	Absorbance values (450-690 nm)	
	Control group	D <sub>3</sub>
0	259.354x10 <sup>-3</sup> * ± 0.27	258.243x10 <sup>-3</sup> ± 0.22
12	321.230x10 <sup>-3</sup> ± 0.19	212.342x10 <sup>-3</sup> ± 0.28
24	398.256x10 <sup>-3</sup> ± 0.33	194.784x10 <sup>-3</sup> ± 0.31
48	429.670x10 <sup>-3</sup> ± 0.41	168.298x10 <sup>-3</sup> ± 0.47
72	472.231x10 <sup>-3</sup> ± 0.32	111.625x10 <sup>-3</sup> ± 0.52

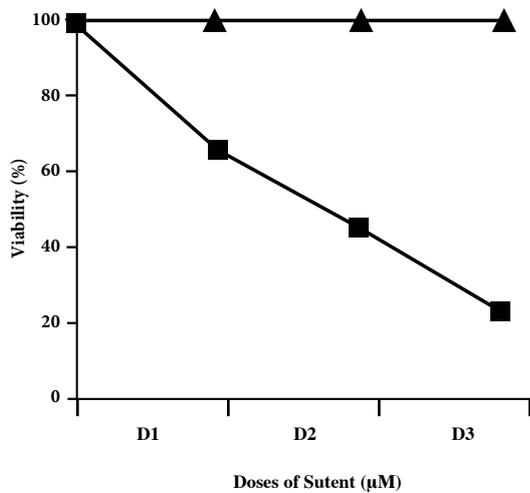
SD: standard deviation. \*Significantly different ( $p < 0.01$ )



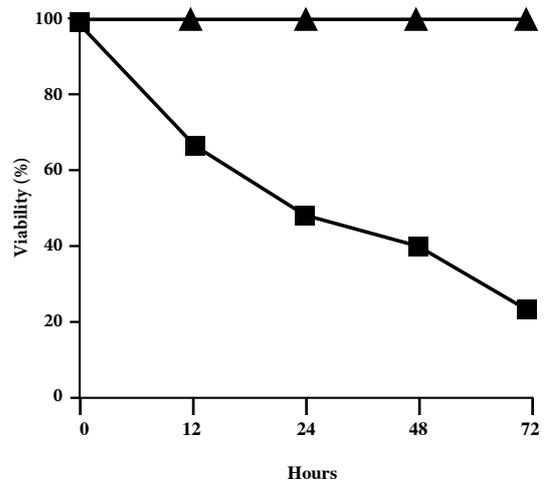
**Figure 1.** Absorbance values of mitochondrial dehydrogenase enzyme activity of HeLa cells treated with three different doses of sunitinib ( $D_1 = 1\mu\text{M}$ ,  $D_2 = 5\mu\text{M}$ ,  $D_3 = 10\mu\text{M}$ ) for 72 h (450-690 nm) ( $p < 0.01$ ).



**Figure 3.** Absorbance values of mitochondrial dehydrogenase enzyme activity of HeLa cells treated with  $D_3$  dose of sunitinib ( $D_3 = 10\mu\text{M}$ ) for 0-72 h (450-690 nm) ( $p < 0.01$ ).



**Figure 2.** Percent viability values of HeLa cells treated with three different doses of sunitinib ( $D_1 = 1\mu\text{M}$ ,  $D_2 = 5\mu\text{M}$ ,  $D_3 = 10\mu\text{M}$ ) for 72 h (the horizontal line shows the control group).



**Figure 4.** Percent viability values of HeLa cells treated with  $D_3$  dose of sunitinib ( $D_3 = 10\mu\text{M}$ ) for 0-72 h (the horizontal line shows the control group and the dropping line the  $D_3$  dose).

**Table 3.** Mitotic index values of HeLa cells treated with  $D_3$  dose of sunitinib ( $D_3 = 10\mu\text{M}$ ) for 0-72 h ( $\pm\text{SD}$ )

Experiment groups Time of exposure (hours)	Mitotic index values (%)	
	Control	$D_3$
12	$7.62 \pm 0.24^*$	$5.00 \pm 0.12$
24	$7.21 \pm 0.51$	$3.44 \pm 0.37$
48	$5.28 \pm 0.22$	$2.42 \pm 0.13$
72	$5.14 \pm 0.21$	$1.10 \pm 0.14$

SD: standard deviation. \*Significantly different ( $p < 0.01$ )

doses was applied to the cells at 0,12,48 and 72 h.

After treatment of HeLa cell culture with  $D_3$ , absorbance values of the control and experimental groups were measured for each hour (Table 2). Statistical analysis showed significant difference between control and experimental groups at 12-72 h ( $p < 0.001$ ) (Figure 3).

According to the absorbance values, while viability of the control group was considered 100%, the viability values of the experimental groups compared with the control group were 66% in 12 h, 48% in 24 h, 39% in 48 h and 23% in 72 h (Figure 4).

According to the mitochondrial dehydrogenase enzyme activity analysis which was performed with administration of sunitinib for 0-72 h it was concluded that the viability of HeLa cells was significantly decreased in a time-dependent manner ( $p < 0.001$ ).

### Apoptosis

#### Morphological evaluation

The apoptotic morphological changes were shrinkage and blebbing of the cell membrane, and nuclear condensation, cell deformation, formations of apoptotic bodies and nuclear breaks into fragments. These apoptotic morphological changes were detected under light and fluorescence

microscope on HeLa cells treated with  $D_3$  of sunitinib for 12, 24, 48 and 72 h.

#### Apoptotic index

For 0-72 h after administration of  $D_3$  dose of sunitinib which was the most effective dose compared to other doses, approximately 200 cells were counted for both control and experimental groups. AI was 3% for the control, 34% for  $D_1$ , 58% for  $D_2$  and 67% for  $D_3$  for the experimental groups (Figure 5). All the differences between control and experimental groups were statistically significant ( $p < 0.001$ ). In addition, a significant difference was noted among experimental groups ( $p < 0.005$ ). AI values of  $D_3$  dose for 0-72 h are shown in Figure 5. Statistically, there was a significant difference between the control and experimental groups ( $p < 0.001$ ).

### Mitosis

#### Morphological evaluation

Late prophase, metaphase, anaphase and telophase of mitosis in HeLa cells were evaluated with Feulgen method and Giemsa staining under light microscope. Administration of  $D_3$  of sunitinib at 72 h caused tripolar metaphase and a plenty of anaphase bridges, a phenomenon that doesn't occur in normal cell division.

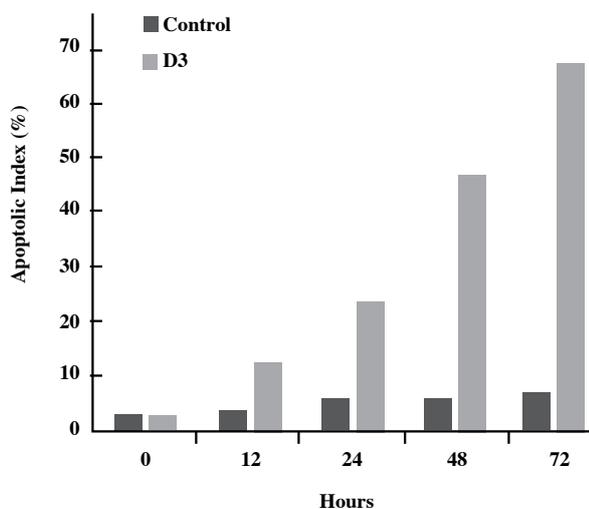
#### Mitotic index

For 0-72 h after administration of  $D_3$  dose of sunitinib which was the most effective dose compared to other doses, 3000 cells were counted for both the control and experimental groups.

MI values of  $D_3$  dose are shown in Table 3. The difference was significant between the control and experimental groups ( $p < 0.001$ ). In addition, statistically significant difference was noted among all experimental groups ( $p < 0.001$ ). The optimal dose of sunitinib was  $D_3$  (MI 1.10%). MI values were significantly different between the control and experimental groups ( $p < 0.001$ ).

## Discussion

In this study, the cytotoxic effects of sunitinib were evaluated with parameters of cell kinetics including mitochondrial dehydrogenase enzyme activity analysis, AI and MI.



**Figure 5.** Apoptotic index values of HeLa cells treated with  $D_3$  dose of sunitinib for 0-72 h and control ( $p < 0.01$ ).

Sutent exerts cytotoxic activity in breast, thyroid and colorectal cancer as well as in metastatic gastrointestinal tumors and metastatic renal cell carcinoma [10-12].

Administration of sutent to human cord endothelial cells brings about apoptosis [13]. In a study which examined different methods of tumor therapy, it was supposed that inhibition of angiogenesis can be an effective anticancer therapy [14].

An *in vitro* study indicated that sutent is an effective drug derived from indole compounds. Sutent inhibited the proliferation of TPC-1 cells in a dose-dependent manner ( $IC_{50}$  224 nM). It targeted tumor cell proliferation and survival directly with suppression of receptor tyrosine kinases (RTK) such as PDGFR, VEGFR, KIT and FLT3. This study showed that sutent was an inhibitor of RET/PTC oncoprotein with  $IC_{50}$  224 nM and was more advantageous because of low toxicity, antiangiogenic activity and oral intake compared with other compounds which were developed for RET/PTC associated papillary thyroid carcinoma (PTC) therapy [15].

It was shown that 10  $\mu$ M of sutent have antiangiogenic activity on U87MC glioma cells and this high dose is not toxic for the healthy glial cells [16].

In our investigation, the optimal cytotoxic dose was 10  $\mu$ M. This dose was applied for 72 h to the cells. Mitochondrial dehydrogenase enzyme activity was detected and as a result, while viability was 100% for the control group, viability of the experimental groups dropped to 23%.

In a study with preclinical evaluation of sutent, based on data derived from breast cancer, this drug exerted intense antiproliferative activity alone or combination with conventional cytotoxic agents (5-fluorouracil, doxorubicin) [17].

In a clinical investigation with sutent administered to patients with gastrointestinal stromal tumor, apoptosis increased 6-fold in all patients receiving the drug compared to pre-treatment levels [18].

In an *in vitro* investigation, it was demonstrated that sutent suppresses cellular proliferation and increases apoptosis in a dose-dependent manner in MV4-11 cell line ( $IC_{50}$  1-10 nM) [19].

In another study which was done on U87 and M059K glioma cells, it was observed that cell pro-

liferation decreased after 48 h exposure to sutent. The proliferation of these cell lines was inhibited by 95% after 10 $\mu$ M of sutent. In addition, a significant increase in apoptosis was observed after 24 h of exposure of these cell lines to sutent [20].

Phosphorylation of CSF-1R receptor that belongs to RTK family and is expressed by NIH3T3 cells, was inhibited with  $IC_{50}$  50-100 nM of sutent. An *in vivo* study on mice, 40 mg/kg/day of sutent for 21 days inhibited tumor growth in bone by 64%. Depending on these findings, it was concluded that this drug can be used as an effective agent for prevention of bone metastasis in breast cancer [21,22].

In an *in vitro* study done on 5637 and TCC-SUP cell lines of human invasive transitional cell carcinoma, sutent blocked cell proliferation at a dose of  $IC_{50}$  9.9  $\mu$ M and  $IC_{50}$  7.5  $\mu$ M, respectively [23].

In our study, administration of  $D_3$  (10 $\mu$ M) of sutent for 72 h increased the AI to 67% vs. 3% in the control group ( $p < 0.001$ ). This increase was thought as an important finding for inhibition of tumor growth.

Also, MI was 1.1% for the experimental groups and 5.14% for the control group after administration of sutent at  $D_3$  for 72 h. These data suggested that the reduction in the number of cells in mitosis due to apoptotic cell deaths brought about the decrease of MI. Again, the decrease in the percentage of living cells with administration of  $D_3$  dose for 72 h seems to support this opinion.

Determination of telomerase activity in tissues with the development of the TRAP method allowed the investigation of the expression of telomerase in a large number of cancers. Studies show that telomerase is the most common determinant of cancer; about 85% of malignant tumors display telomerase activity, suggesting that telomerase is a very important indicator in the diagnosis of cancer [24].

In our investigation, MI studies showed a relatively large number of cells with anaphase bridges and a small number of cells with tripolar metaphase with administration of  $D_3$  dose for 72 h. Relative to this unexpected finding, some studies had shown that anaphase bridges occur due to telomere shortening or degradation [25-29]. Also, the authors of a study reported a complete relationship between reduction

of telomere length and frequency of endogenous bridges [30].

In the present study, changes in the cell cycle of HeLa cells caused by sunitinib were investigated and, to our knowledge, this is the first investigation on this topic. Administration of sunitinib to HeLa cell culture with optimal dose (10  $\mu$ M), especially for 72 h, caused significant increase of AI and decrease of MI. Also the existence of anaphase bridges and tripolar metaphase in 72 h, but not in previous hours, suggested that there was a chromosome instability caused by telomerase shortening. This assumption can lead to new investigations about the use of sunitinib for telomerase-targeted therapy.

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