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Sorefenib inhibits the expressions of heat shock protein70 gene on multiple myeloma (RPMI-8226) and plasma cell leukemia (ARH-77) cell lines

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ÖZET

Sorefenib ısı şok protein70 genini multiple myelom (RPMI-8226) ve plazma hücreli lösemi (ARH-77) hücre hatlarında inhibe eder

Sorafenib solid yapıdaki tümörlerde kullanılan antikanser ilaçtır. Bu ilaç hücrede pek çok tirozin kinazı inhibe eder ve hücreyi apopitoza yönlendirir. Multipl myeloma ve plazma hücreli lösemi hastalık olarak plazma hücreli diskraziler içerisindedir. Halen her iki hastalığın tedavisinde de zorluklar vardır. Isı şok proteinler hücre için önemli olup protein katlanmasında, hücrelerin stersen korunmasında rolü alırlar. Isı şok proteinler birçok kanser hücrelerinde artmış olarak bulunur. Bu proteinlerin aşırı yapımı hücre büyümesi ve metastazında önemlidir. Çalışmamızda sorafenibin sitotoksik etkisi multipl myelom ve plazma hücreli lösemi hücre hatlarında tripan blue ve MTT yöntemleri ile bakılmıştır. MTT yöntemi ile RPMI-8226 hücrelerinde LD50 dozu 12 µM, ARH-77 hücrelerinde 9 µM bulunmuştur. Tripan blue ile yapılan hücre canlılık oranları analiz sonuçları MTT sonuçlarını desteklemektedir. Multipl myelom hücre hattında %89 oranında, plazma hücreli lösemi hücre hattında %80 oranında canlı hücre saptanmıştır. Çalışmamızda ısı şok protein70 ve 90 gen ekspresyon sonuçları da aynı zamanda bu hücre hatlarında çalışılmıştır. Sorafenibin ısı şok protein70 gen ekspresyonunu her iki hücre hattında da inhibe ettiği saptanmıştır. Bu sonuç, sorafenibin sitotoksik etkisinin apopitozu multipl myelom ve plazma hücreli lösemi hücre hatlarında ısı şok protein70 geni üzerinden etkileyerek oluşturduğunu göstermektedir.

Anahtar Kelimeler: *orefenib, multipl myelom, plasma hücreli lösemi, ısı şok protein70, ısı şok protein90.*

SUMMARY

Sorafenib is an anticancer drug used in solid tumors. It inhibits many tirosine kinases in the cell and causes the tumor cell undergone to apopitosis. Multiple myeloma and plasma cell leukemia both are plasma cell dyscrasias and have limited treatment procedures. Heat shock proteins are the important part of the cell's machinery for protein folding and help to protect cells from stress. Heat shock proteins are high in tumor cells in some cases. The over-expressions of these proteins increase the tumor growth and metastatic potential. In our study, the cytotoxic effects of Sorafenib were analyzed on multiple myeloma and plasma cell leukemia cell lines with trypan blue and MTT analyses. The LD50 value is found as 12 µM in RPMI-8226 cells and 9 µM in ARH-77 cells by using MTT cell proliferation assay. Cell viability assay findings with trypan blue were supported the MTT analyses results. In multiple myeloma cell line 89% viability was found with sorafenib. In plasma cell leukemia cell line, it was found as 80%. Also the gene expression analyses of heat shock proteins70 and 90 were studied on these cell lines in our study. Sorafenib inhibited heat shock protein70 gene expressions in these two cell lines in a dose- and time-dependent manner. So, sorafenib has cytotoxic affect by inducing apopitosis on heat shock protein70 gene in multiple myeloma and plasma cell leukemia cell lines.

Key words: *Sorefenib, multipl myeloma, plasma cell leukemia, heat shock protein70, heat shock protein90.*

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Introduction

Sorafenib is an anticancer drug, which inhibits kinases. As a drug, it is used in the treatment of primary kidney cancer (advanced renal cell carcinoma), advanced primary liver cancer (hepatocellular carcinoma), and radioactive iodine resistant advanced thyroid carcinoma. In a tumor cell, it inhibits the serine–threonine kinases (Raf-1 and B-Raf) and the receptors of tyrosine kinases (vascular endothelial growth factor receptors-VEGFRs and platelet-derived growth factor receptor β-PDGFR-β) (1). Also it stimulates caspase-3 on apopitotic pathway (2). Sorafenib induces autophagy in a tumor cell. Autophagy suppresses tumoral growth (3, 4).

Plasma cell dyscrasias, originated from plasma cells [such as multiple myeloma (MM) and plasma cell leukemia (PCL)] have high mortality rates despite of new drugs. MM and PCL participate also in chronic lymphoproliferative disorders (5). PCL is an aggressive form of MM. It is characterized by high levels of abnormal plasma cells circulating in the peripheral blood. Due to aggressiveness of the disease, it is associated with a poor prognosis. Current treatments for PCL are the same as those for myeloma. The resistance in cancer treatment generally occurs in some of these cases. So, more intensive treatments (using combinations of chemotherapy drugs, steroids and new agents) may be considered (6). In literature, sorefenib was used successfully in the treatment of MM recently. As a drug it uses in phase II studies in relapsed-refractory MM (RR-MM) treatments (7, 8).

Heat shock proteins (HSPs) are produced by cells in response to exposure to stressful conditions. HSP70 and HSP90 are the most widely studied HSPs (9). They are highly expressed in cancerous cells. Both of them are essential to the survival of cancer cells (10). The specific inhibition of HSP90 in a cancer cell affects on the Raf kinase signaling pathway. A Raf kinase inhibitor may enhance the antitumoral effect of sorafenib (11). Recently; Jakubowicz-Gil et al. demonstrated that sorafenib and quercetin are very effective programmed cell death inducers in T98G and MOGGCCM cells, especially in cells with blocked expression of HSPs (12).

So, the cytotoxic effect of sorafenib was analyzed on MM cell line (RPMI-8226) and PCL cell line (ARH-77) by using trypan blue cell viability assay and MTT cell proliferation assay. Also the expression differences were analyzed with different concentrations of Sorafenib of in HSP 70-90 genes. According to our results, Sorafenib has cytotoxic affect on these two cell lines. Also it decreases HSP-70 gene expression. These results represent us the role of this anticancer drug on HSP70 gene which has role on cell apopitosis in tumor cells.

Material and Methods

This project was granted by Gülhane Military Medical Academy, Health Science Institute (AR-2012/23) with the Gülhane Military Medical Academy, Ethical Committee decision (May 31-2012/187).

Preparing Sorafenib Solutions

Sorafenib (BAY 43-9006, Nexavar™) was obtained directly from the producing company. Sorafenib solution is dissolved in Fetal Bovine Serum-FBS (BiochromAG, Germany) in different concentrations (0.1 µM, 1 µM, 2.5 µM, 5 µM, 10 µM, 20 µM, 40 µM, 80 µM, 160 µM).

Cell Culture

MM [RPMI-8226 (ATTC No: CCL-155) Human multiple myeloma] and PCL [ARH-77 (ATCC No: CRL-1621) Human plasma cell leukemia] cell lines are obtained from Gülhane Military Medical Academy, Health Science Institute, Cancer Research Center. Both cell lines are incubated in RPMI-8226 1640 (Sigma-Aldrich-R8758) including 10 % (v/v) FBS (BiochromAG, Germany) and 1% (v/v) penicillin and streptomycin (Biological Industries, Israel) (37˚C, 5% CO2) (Heraus incubator, Henau, Germany) (13).

Cell viability assay with trypan blue

Trypan blue (Sigma Aldrich Co. 302643) as a stain was used in procedure for viable cell counting. Trypan blue was diluted at 0.8 mM in PBS. It was mixed with the cells 1:1. In this method, live (viable) and dead (non-viable) cells were counted on hemocytometer (14). Trypan blue cell viability assay was applied in the different concentration of Sorafenib after the 24-hour incubation.

MTT Cell Proliferation Assay

For cell viability and cell proliferation, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5- diphenyltetrazolium bromide; thiazolyl blue) assay was applied in the different concentration of Sorafenib after the 24-hour incubation (Sigma-Aldrich/In Vitro Toxicology Assay Kit, MTT based). According to the instruction manual, we noticed to do analyses with 3x104 cells in each cell culture flask. In each type of cultured cell, Lethal Dose 50 (LD50) was found with Sorafenib application. Also time dependent LD50 values (in 48 and 72-hour) were found with Sorafenib with the dosages of LD50 and low dosages [% proliferation = (control-research well / control)x 100].

RNA Isolation and cDNA Synthesis

RNA isolation was performed from the each example of RPMI-8226 and ARH-77 cell lines. Four samples (One for control, one for 24-hour, one for 48-hour and one for 72- hour) were used for RNA isolation in each condition (Roche RNA isolation kit). RNAs obtained from four different groups in each condition were used in cDNA synthesis (Revert Aid cDNA synthesis kit). The quality of c-DNA was controlled with 2% agarose gel.

Real-time Polymerase Chain Reaction (RT-PCR) for Gene Expression Analyses

RT-PCR was used in the cDNAs obtained in our experiments for HSP-70 and HSP-90 gene expression analyses. The primers for HSP 70 and HSP90 genes were selected from http://pga.mgh.harvard.edu/primerbank/ web pages. As internal control, beta-actin housekeeping gene was used [5'-GTC CCT CAC CCT CCC AAA AG-3' (forward) and 5'-GCT GCC TCA ACA CCT CAA CCC-3' (reverse)]. In RT-PCR, following conditions were used; 95ºC for 10'', 56ºC for 15'', 72ºC for 15''- 45 cycle (Roche Light Cycler1.5). Each reaction was performed as 20 µl (10 µl 2x SYBR, 5 µl c DNA, 0.5 µl primer, 3 µl d H2O). Each sample was studied for 8 times for proper statistical results. Results were analyzed by "Roche Light Cycler1.5 software".

Statistical Analyses

The LC50 nomination method was used for having LD50 values. The same methodology was used for the sorafenib's time dependent LD50 difference either. For evaluating the results of RT-PCR analyses, Student t test was used for comparison.

Results

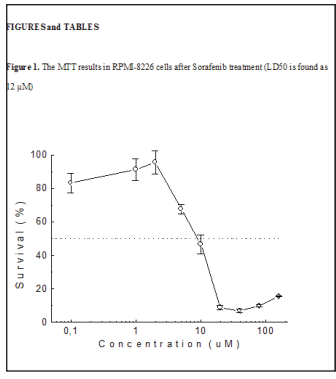

Figure 1. The MTT results in RPMI-8226 cells after Sorafenib treatment (LD50 is found as 12 µM)

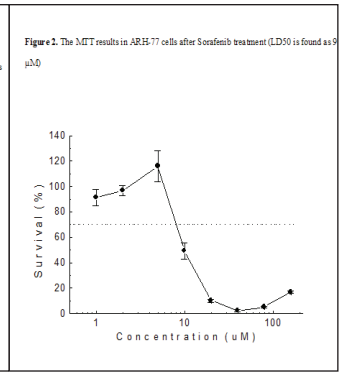

Figure 2. The MTT results in ARH-77 cells after Sorafenib treatment (LD50 is found as 9 µM)

In our experiment, the LD50 value of sorafenib was found as 12 µM in RPMI-8226 cells and 9 µM in ARH-77 cells by using MTT cell proliferation assay (Figure 1 and 2). Also, the time releated lethal dosage of sorafenib were found on MM and PCL cell cultures. The effect of Sorafenib for 48 and 72-hour was detected on RPMI-8226 cells by MTT Assay. Due to results, the effective dosage of Sorafenib for 48 and 72-hour was less than 1 µM (Figure 3). On ARH-77 cells, the lethal dosage of Sorafenib was found 7.5 µM for 48 hours. The lethal dosage of Sorafenib for 72 hours was found less than 1 µM (Figure 4).

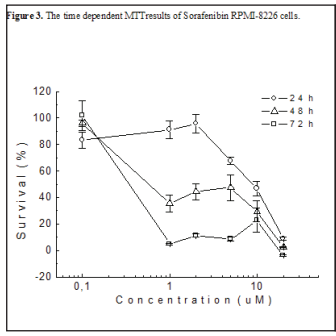

Figure 3. The time dependent MTTresults of Sorafenibin RPMI-8226 cells

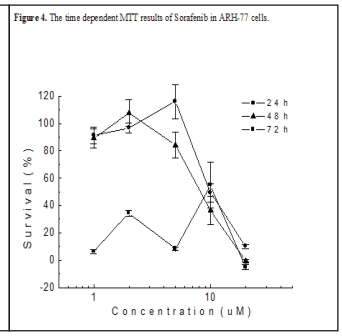

Figure 4. The time dependent MTT results of Sorafenib in ARH-77 cells.

Also, the effect of sorafenib on the gene expressions of HSP 70-90 genes were detected either. For the RPMI-8226 cells, the result of gene expression levels of HSP-70 gene in

Table 1. The RT-PCR results of Sorafenib on *HSP-70* gene expression levels

	RPMI-8226-K	**RPMI-8226-24**	**RPMI-8226-48**	**RPMI-8226-72**
HSP-70 gene expression	0,003±0,0001	0,0004±0,0002	0,002±0,0004	0,001±0,0002
P value		p<0.05	p<0.05	p<0.05
	ARH-77-K	**ARH-77-24**	**ARH-77-48**	**ARH-77-72**
HSP-70 gene expression	0,001±0,0006	0,0006±0,000006	0,0004±0,0003	0,003±0,0003
P value		p<0.05	p<0.05	p<0.05

RPMI-8226-K: RPMI-8226 cells- control group (no treatment)

RPMI-8226-24: RPMI-8226 cells- 24 hours group (24 hours later after Sorafenib treatment)

RPMI-8226-48: RPMI-8226 cells- 48 hours group (48 hours later after Sorafenib treatment)

RPMI-8226-72: RPMI-8226 cells- 72 hours group (72 hours later after Sorafenib treatment)

ARH-77-K: ARH-77 cells- control group (no treatment)

ARH-77-24: ARH-77 cells - 24 hours group (24 hours later after Sorafenib treatment)

ARH-77-48: ARH-77 cells - 48 hours group (48 hours later after Sorafenib treatment)

ARH-77-72: ARH-77 cells - 72 hours group (72 hours later after Sorafenib treatment)

the control group was found 0,003 fold in RPMI-8226 cells. The gene expression of HSP70 gene was found 0.0004 fold in 24-hour sorafenib treated group (Table 1). In 48 and 72-hour sorafenib treated groups, the gene expressions of HSP70 were found as 0.002 fold and 0.001 fold on RPMI-8226 cells respectively (Table 1). The gene expression of HSP-70 gene was found 0.0001 fold in the control group on ARH-77 cells. The gene expression levels of HSP-70 was found 0,0006 fold in 24-hour group. In 48 and 72-hour sorafenib treated groups, the gene expressions of HSP-70 were found as 0,0004 fold and 0,003 fold on ARH-77 cells respectively. All the results obtained in 24-hour, 48-hour and 72-hour group were statistically significant due to control group in RPMI-8226 and ARH-77 cell lines (p<0.05) (Table 1).

In HSP90 gene expressions, no significant elevation was observed in treatment with sorafenib (24-hour, 48-hour and 72 hour) in RPMI-8226 and ARH-77 cell lines (The data not shown).

Discussion

Sorafenib is a specific molecular inhibitor of several tyrosine protein kinases, such as VEGFR, PDGFR and Raf family kinases (1, 15). The cytotoxic activity of this drug induces tumor cell death on tumors (1, 4, 15). As known, MM and PCL have no sufficient treatment procedure (5, 16). Although advancements have been made in the treatment (average life expectancy has risen from 3 to 6 years) this still remains an incurable disease, with nearly all patients relapsing after initial treatment (17). So, new drugs including sorafenib are using in clinical trials. In 2014, Southwest Oncology Group (SWOG) published the results of Phase II trial in RR-MM patients in the uses of sorafenib as a single agent. They pointed that further researches should focus on combination therapy of sorafenib with standard treatments in selected patients with RR-MM (8). Yordanova et al. reported a study including phase II clinical trial results in total 11 recurrent/refractory MM.patients. According to the results, sorafenib treatment was found as effective in two patients who achieved a partial response and a continuous stable disease with duration of 24.4 months and 6.9 month, respectively (18). In our study, the cytotoxic affect of sorafenib was tested on MM and PCL cell lines for finding the effectiveness of this drug on these malignancies. In our study, the lethal dosage and the time releated lethal dosage of sorafenib were found on MM and PCL cell cultures. These results supported the cytotoxic affect of sorafenib on these two cell lines. After 24-hour treatment, the lethal dosage of sorafenib was found as 12 µM. After 48 and 72- hour treatment, the lethal dosage came down to 1 µM both in RPMI-8226 cells (Figure 1, 3). This gives rise to think cronic usage of sorafenib causes toxic effects. Meanwhile, it represents us the dosages can be lower down in the usage of sorafenib for long times. As known, metronomic chemotherapy (MC) refers to the close administration of a chemotherapeutic drug for a long time with no extended drug-free breaks. It was developed to overcome drug resistance, partly by shifting the therapeutic target from tumor cells to the tumor vasculature, with less toxicity. Gnoni et al. also pointed that vinorelbine, cyclophosphamide, capecitabine, methotrexate, bevacizumab, etoposide, gemcitabine, sorafenib, everolimus and temozolomide may be use in clinical studies in MM as chemotherapy drugs with a metronomic schedule (19). So, our experiment is important for having low toxicity in sorefenib in possible MM treatment. Our results also support Gnoni et al.'s findins.

In 24-hour treated group, the lethal dosage of sorafenib was found as 9 µM in ARH-77 cells. After 48 hours treatment the lethal dosage lowered down to 7.5 µM. In 72-hour treatment, the lethal dosage came down to 1 µM (Figure 2,4). These findings represent the resistance of the PCL cells to sorafenib. This can be occured because of the short doubling time of PCL cells. It known that tumor cell turn over is nearly half in PCL

cells due to MM cells (20). This finding can explain the different sitotoxic effect of sorafenib dependent to time between two cell lines. On the other hand, the increase in the cell proliferation after 72 hours treatment can be seen in figure 4. This finding also represents the proliferation of possible robust cells after the chronic usage. In long time usage, the proliferation pattern of PCL cells can be explained by the drug resistance (Figure 4) (19, 21). Cell death was characterized by phosphatidylserine exposure, ΔΨm loss, cytochrome c release and caspase activation, hallmarks of apoptosis. Ramírez-Labrada et al. analyzed cell death induced by sorafenib in MM cell lines and in plasma cells from MM patients by using RT-MLPA and quantitative PCR, protein levels and functionality by Western blot and flow cytometry and gene silencing with siRNA methodology. Sorafenib treatment increased levels of Puma gene at mRNA and protein level and gene silencing with siRNA confirmed a relevant role for Puma in the induction of apoptosis in MM cell lines (22). This result represents the possible role of sorafenib in MM cells at gene level. There may be another gene or genes which are affected by sorafenib in MM or PLC tumor cells. So, the roles of HSP genes were analyzed in our experiment on plasma cell dyscrasias in vitro.

HSPs can be induced by many environmental factors. Especially they arise in the cells in troubled state such as high temperature. At this stage HSP acts as an intracellular chaperone. They bound to the proteins and enhance the effects of the proteins by changing their three-dimensional structures (23). The gene expression alterations of HSP70 and HSP90 genes were found in many cancer cells like breast, colon and pancreas. Also, HSP70 and HSP90 participate in many key processes in oncogenesis such as self-sufficiency in growth signals, stabilization of mutant proteins, angiogenesis, and metastasis. They act like antiapoptotic chaperones (23, 24, 25). In our study, HSP70 and HSP90 gene expressions were studied in MM cells and PCL cell lines. Due to our results, sorafenib HSP70 gene expression reduced in RPMI-8226 and ARH-77 cells compared to control group in 24-hour group. In the long-term usage of Sorafenib in RPMI-8226 cells decreased the expression of HSP70 gene in 48 and 72-hour groups (Table 1). These results may represent the possible role of serafenib on HSP70 gene. Sorefenib inhibits the expression of HSP70 gene in these two cell lines. In many types of cancers, HSP70 abnormal gene expression levels were reported. In breast cancer patients, HSP70-2 was over expressed and was involved in malignant properties of breast cancer. In esophageal cancer, reduced HSP27 and HSP70 gene expressions were found as associate with poor survival in patients with esophageal cancer, especially esophageal squamous cell carcinoma (26, 27). So, Kumar et al. noticed that Hsp70 may be a target molecule in the development of cancer therapeutics (28). Our results represent the inhibitory role of sorefenib on MM and PCL cell lines which represents antitumoral affect of sorefenib at gene level. No specific finding was observed in HSP90 gene expression analyses in our experiments.

In a recent article, Gentile at al. pointed out that, sorafenib had a good safety profile but minimal anti-myeloma activity as a single agent in relapsed/refractory patients. Results of phase II trials, evaluating sorafenib combined with new drugs, such as bortezomib and lenalidomide are eagerly awaited (7). Our results also supported these findings. The inhibition of HSP-70 gene expression may represent a possible role of sorefenib on this gene.

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