

Cytotoxic Activity of Apixaban on HeLa Cells: An In vitro Study

Apiksabanın HeLa Hücreleri Üzerindeki Sitotoksik Aktivitesi: in vitro Bir Çalışma

Research Article

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ABSTRACT

Apixaban is a new oral anticoagulant which is commonly used in the prophylaxis and treatment of systemic embolism and deep vein thrombosis. Venous thromboembolic events are important risk factors in cancer patients. Several studies have suggested that anticoagulant drugs may have an effect on tumor development and progression. In this study, we aimed to investigate the cytotoxic effect of apixaban on a cancer cell line HeLa cells derived from human cervical cancer. The viability of HeLa cells was examined at 24 and 48 hours of incubation and apixaban showed a cytotoxic effect in the highest dilution. In other dilutions, cell viability was similar to the control group. These results show that apixaban may reduce proliferation of cancer cells at high concentrations.

Key Words

Apixaban, new oral anticoagulant, venous thromboembolism, cytotoxicity, HeLa cells.

ÖZ

Apiksaban, sistemik emboli ve derin ven trombozunun profilaksisi ve tedavisinde yaygın olarak kullanılan yeni nesil bir oral antikoagülandır. Venöz tromboembolik olaylar, kanser hastalarında önemli birer risk faktörüdür. Bazı çalışmalar, antikoagülan ilaçların tümör gelişimi üzerinde etkisi olabileceğini göstermektedir. Bu çalışmada, insan serviks kanseri kaynaklı hücre hattı HeLa hücreleri üzerinde apiksabanın sitotoksik etkileri incelendi. Hücre canlılığı inkübasyonun 24. ve 48. saatlerinde ölçüldü ve apiksaban en yüksek dilüsyonda toksik etki gösterdi. Diğer dilüsyonlar kontrol grubu ile benzer bulundu. Bu sonuçlar, apiksabanın yüksek dilüsyonda kanser hücrelerinin proliferasyonunu azaltabileceğini göstermektedir.

Anahtar Kelimeler

Apiksaban, yeni nesil oral antikoagulan, venöz tromboembolizm, sitotoksosite, HeLa hücreleri.

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INTRODUCTION

Thromboembolic diseases are the leading causes of death in developed countries. Oral anticoagulants are widely used for prevention of cardiac and venous thromboembolism (VTE). During the past six decades, vitamin K antagonists, such as warfarin, have been extensively used for anticoagulant therapy [1]. However, novel agents have recently been developed, as the use of vitamin K antagonists is limited due to the food interactions and need for monitoring. New oral anticoagulants (NOACs) are mainly activated factor Xa (FXa) or thrombin [1]. Apixaban is a highly selective and well-tolerated oral factor Xa inhibitor [2]. It has predictable pharmacokinetic properties and fixed-dose regimen. Apixaban has been widely used worldwide after the approval of the United States Food and Drug Administration (FDA) and by the European Medicine Agency (EMA) [1].

Thromboembolic disorder is a serious risk factor in cancer patients [3]. Acute VTE is the second leading cause of death after the malignancy itself. Recurrent VTE and bleeding complications are high risk factors in anticoagulant treatment for acute VTE in cancer patients [4,5]. Therefore, developing new modalities is highly important, and NOACs have the potential to be alternative options for these patients [4]. In addition to their benefits to prevent VTE, anticoagulant drugs may have an inhibitory effect on tumor development and progression [6]. Recently, a FVIIa inhibitor has been found to be an inducer of apoptosis in tumor cells through activating caspase cascade [7]. However, there is only a few data suggesting that anticoagulant drugs may have an inhibitory effect on cancer cells.

Cervical cancer is one of the primary causes of cancer-related deaths and new cases have been increasing every year all over the world [8]. Cervical adenocarcinoma (ADC) has an increasing prevalence unlike squamous cell-carcinoma (SCC) [9]. Cervical adenocarcinoma comprises nearly 20 to 25% of the all cervical malignancy in developed countries [10]. Three types of ADC occur in the cervix. Mucinous adenocarcinoma is the most common type of ADC in the cervix. Endometrial ADC is a type that develops in the lining of the uterus. Clear-cell ADC may occur in daughters of women who used diethylstilbestrol during

pregnancy [9]. HeLa cells are a line of human cells derived from cervical cancer [11]. These cells were isolated from a woman named Henrietta Lacks from her cervical tumor in 1951, which is currently known as the HeLa cells [12]. The HeLa cell line is an endocervical adenocarcinoma cells [13]. This cell line was the first cell line to survive outside the human body and grow. They have shown high durability and versatility and have been used for applications such as drug discovery, virus studies, and cancer researches [11]. In the present study, we aimed to investigate cytotoxic activity of apixaban on the HeLa cell lines derived from human cervical cancer.

MATERIALS and METHODS

Cell Culture and Treatment of Test Material

The HeLa cells were placed in 96-well culture plates (Greiner Bio-One, Germany) at an initial density of 30.000 cells/mL in six replicates and incubated in the Dulbecco's Modified Eagle's Medium (DMEM)/Ham's F12 (Biowest Inc., Nuaillé, France) supplemented with 10% fetal bovine serum (FBS) (Biowest Inc., Nuaillé, France) in a humidified atmosphere of 95% air and 5% CO₂ for 12 hours at 37°C. Following incubation, the cells were treated with five dilutions of the test material [Eliquis (apixaban, 5 mg)TM]. Test material was prepared in (DMEM)/Ham's F12 supplemented with 1% dimethyl sulfoxide (DMSO) (Sigma Chemical Co., St. Louis, MO), which is a safe organic solvent to prepare solutions used in cell culture tests. Stock solution was prepared as 0.30 g/20 mL for the initial dose, according to the molecular weight of the agent (apixaban: 459.506 g/mol). Stock solution was kept at room temperature for 24 hours and, then, filtered using 0.8 µm and 0.2 µm filters before use. Five dilutions of test material were prepared in (DMEM)/Ham's F12 medium containing 10% FBS by serial dilution [Dilution I (stock solution): 30 µM; Dilution II: 15 µM; Dilution III: 7.5 µM; Dilution IV: 3.75 µM; Dilution V: 1.875 µM].

Cell Morphology

Morphological changes of the cells were examined under an inverting microscope (IX70 Olympus, Japan). All groups within test series were compared with the control group separately for each evaluation period.

Cell Viability

The cell viability was determined by MTT assay. The viability of HeLa cells was examined at 24 and 48 post-incubation hours. At each evaluation period, the culture medium was removed, and 100 μ L DMEM/F12-without FBS containing 12.5 μ L MTT were added into each well. Culture plates were covered with aluminum foil and cells were, then, incubated in the dark for four hours. At the end of the incubation period, MTT solution was removed from the wells and 100 μ L isopropyl alcohol was added. The absorbance at 560 nm was measured using an ultraviolet (UV)-visible spectrophotometer (EZ Read 400 Microplate Reader, Biochrom).

Statistical Analysis

Statistical analysis was performed using the SPSS version 23.0 software (IBM Corp., Armonk, NY, USA). Descriptive statistics were expressed in mean values \pm standard deviation (SD). The Kolmogorov-Smirnov test was used for the normality test. The analysis of variance (ANOVA) was used to compare the means of more than two

groups. A *post-hoc* test (Tukey test or Tamhane's test) was used to examine significant differences between the groups. A *p* value less than 0.05 was considered statistically significant.

RESULTS and DISCUSSION

Cell Morphology

At 24-hour incubation, the cells exposed to dilution I of apixaban showed a significant difference, compared to normal fibroblastic morphology. The cells displayed degeneration as nuclear condensation and rounded cell morphology. Cells in dilutions II, III, IV, and V demonstrated a similar fibroblastic morphology with the control cells (Figure 1a-f).

At 48 hours, the cells did not show significant morphological changes, compared to the cells at 24 hours in all dilutions of the test material.

Cell Viability

The viability of HeLa cells was examined for the test material in different dilutions and compared with the control group at 24 and 48 hours



Figure 1. Morphological appearance of HeLa cells exposed to apixaban at 24 hours of incubation. Dilution I (a); Dilution II (b); Dilution III (c); Dilution IV (d); Dilution V (e); Control (f) (10 \times).

after incubation (Figure 2). Apixaban showed a cytotoxic effect in the highest dilution (Dilution I).

In other dilutions cell viability is similar with control group except for dilution 4. It can be said that initial density of cells in this dilution is higher than in the other dilutions.

The Tukey test or Tamhane's test was performed to show the difference between the dilutions and control group (Table 1). These result indicated that apixaban might reduce proliferation of cancer cells in high dilutions.

The ANOVA was performed to evaluate significant differences among all dilutions at 24 and 48 hours and *p* value was obtained. The results showed that cell viability was statistically significant in all dilutions between 24 and 48 hours, except for Dilution I (Table 2). This result indicated that Dilution I had a cytotoxic activity both at 24 and 48 hours of incubation.

Apixaban [1-(4-methoxyphenyl)-7-oxo-6-[4-(2-oxopiperidin-1-yl) phenyl]-4,5,6,7-tetrahydro-1H-pyrazolo[3,4-c]pyridine-3-carboxamide - $C_{25}H_{25}N_5O_4$] is a direct oral FXa inhibitor which has rapid absorption and 12-hour half-life [14]. It has good bioavailability, low clearance, and a small

volume of distribution and it shows low drug-drug interactions. Apixaban is highly selective for the prevention and treatment of thromboembolic diseases [15]. As thrombin plays a key role in the generation of an occlusive thrombus, inhibition of thrombin activity becomes a major target in the developing of novel antithrombotic agents. The direct FXa inhibitors, such as apixaban, inhibit thrombin production by blocking the FXa in the blood coagulation cascade. Apixaban is a highly potent, reversible, and active-site inhibitor of human FXa, with a K_i of 0.08 nM at 25°C and 0.25 nM at 37°C in the FXa tripeptide substrate assay [16]. Analysis of enzyme kinetics shows that apixaban act as a competitive inhibitor of FXa binding its active site. The reversibility of FXa inhibition is demonstrated by the recovery of FXa activity at 37°C. Apixaban has over 30.000-fold higher selectivity for FXa, compared to other human coagulation proteases and related enzymes [17].

Apixaban is a pyrazole-based drug and it has been reported that the pyrazoles show anticancer activity by inhibiting topoisomerase IIa [18]. Deoxyribonucleic acid (DNA) topoisomerase has been recognized as an important target in anticancer drug discovery [19]. Topoisomerases (I and II) are essential nuclear enzymes that

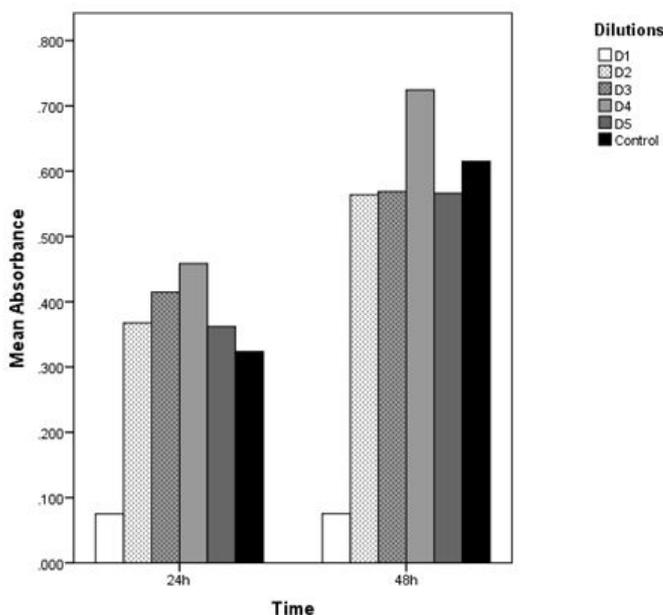


Figure 2. Comparison of cell viability at 24 and 48 hours in different dilutions.

Table 1. Cell viability of the test material at 24 and 48 hours in each dilution compared to control group.

Time	Dilutions	Mean Absorbance	Standard deviation	p
24 h	D1	0.075	0.0007	<0.001*
	D2	0.367	0.054	0.692
	D3	0.415	0.047	0.050
	D4	0.458	0.061	0.001*
	D5	0.362	0.054	0.793
	Control	0.324	0.066	-
48 h	D1	0.075	0.009	<0.001*
	D2	0.564	0.087	0.996
	D3	0.569	0.105	1.000
	D4	0.725	0.119	0.764
	D5	0.566	0.123	1.000
	Control	0.615	0.076	-

p values compared to control group

Table 2. Comparison of cell viability of test material at 24 and 48 hours for each dilution.

Dilutions	Time	Mean Absorbance	Standard deviation	p
D1	24	0.075	0.007	0.916 >0.05
	48	0.075	0.009	
D2	24	0.367	0.054	0.001 <0.05*
	48	0.564	0.087	
D3	24	0.415	0.047	0.008 <0.05*
	48	0.569	0.105	
D4	24	0.458	0.061	0.002 <0.05*
	48	0.725	0.119	
D5	24	0.362	0.054	0.004 <0.05*
	48	0.566	0.123	
Control	24	0.324	0.066	0.000 <0.05*
	48	0.615	0.076	

maintain the topological changes of DNA and play key role in transcription, replication, and chromosome segregation [20]. Anticancer drugs are designed as topoisomerase I and II inhibitors [19]. These drugs are classified into two groups: topoisomerase II poisons and topoisomerase II catalytic inhibitors. Topoisomerase II poisons stabilize the cleavable complex of topoisomerase II with DNA by constituting the covalent ternary complex of drug/agent-cleaved G segment, and enzyme release and stimulate apoptosis.

Topoisomerase catalytic inhibitors play role in any of the other step of catalytic cycle. Recently, pyrazole-based new agents designed, synthesized and the cytotoxic effects on different cancer cell lines have been studied [21].

The possibility of VTE being seen is very high in cancer patients and metastasis also increases VTE risk [3]. The increase of VTE risk is even higher in metastasis of aggressive types of cancers. Besides VTE, patients with cancer have several

bleeding risk factors (i.e., chemotherapy-induced thrombocytopenia, disseminated intravascular coagulation and renal or liver impairment). Cervical cancer is one of the most common malignant tumors affecting women [22]. Venous thromboembolism is also a life-threatening condition which occurs as a complication of cervical cancer [23]. Therefore, effective and safe anticoagulant therapy is of utmost importance in cancer patients and NOACs may be an option for these patients.

Apixaban has been recommended as an alternative to low-molecular-weight heparin for cancer patients with VTE [24]. In an animal model, it was shown that the seeding of tumor metastases was a coagulation-dependent process [25]. In addition, an animal experimental study showed that tissue factor pathway inhibitor, a physiological FXa inhibitor, and the nematode anticoagulant protein rNAPc2 inhibited primary and metastatic tumor growth in mice [26]. Amblyomin-X, a Kunitz type FXa inhibitor, showed cytotoxic effects in cancer cells through inducing apoptosis and promoting cell cycle arrest [27]. In addition, secreting procoagulant and anti-fibrinolytic factors from cancer cells were shown to cause endothelial damage by inflammatory process [28]. The molecular mechanism underlying the link between anticoagulant drugs and tumor biology are not known exactly [29]. Therefore, more definitive and comprehensively experimental studies are needed to investigate this correlation.

As apixaban has a lower bleeding profile different from the other NOACs and it has a possible inhibitor effect on cancer cells [30], it can be a good choice for cancer patients. In a study, cytotoxic effects of synthesized lactam derivatives and rivaroxaban were investigated on HCT-116, HeLa, and HEK-293 cells lines [31]. In another study, *in vitro* effects of apixaban on five different cancer cell lines were investigated [6]. In this study, cell proliferation and mortality were investigated on OVCAR3 (ovarian cancer), MDA MB 231 (breast cancer), CaCO-2 (colon cancer), LNCaP (prostate cancer), and U937 (histiocytic lymphoma) in apixaban-treated cultures. Reduced proliferation was seen in three cancer cell lines

(OVCAR3, CaCO-2 and LNCaP) and increased cell mortality was seen in four cancer cell lines (OVCAR3, MDA MB 231, CaCO-2, and U937). Similar to our study, the increased cancer cell mortality was found during high-dose apixaban incubation[6]. In our study, direct cytotoxic effect of apixaban on cervical cancer cell line was shown only high-dose exposure of apixaban.

Nonetheless, our study has some limitations. First, the experiments were performed in a cell culture milieu which contained 10% fetal bovine serum which induces proliferation of cell, and this may be explained increased cell number at 48 hours of incubation period. The other one, cell migration, gene expression and matrix metalloproteinase in cancer cell lines was not investigated in our study, only cytotoxic data was available.

In conclusion, cytotoxic activity of apixaban on HeLa cells was investigated an *in vitro* cervical tumor model. Our results showed that apixaban may reduce proliferation of cancer cells in high dilutions at 24 and 48 hours. We believe that investigation of cytotoxic effects of this drug should be considered a preliminary study and more detailed studies are needed to explain the cytotoxic mechanism of the drug. To the best of our knowledge, this is the first study in the literature about apixaban cytotoxicity on HeLa cells. Experimental studies should be performed to reveal further data for effect mechanism of apixaban on HeLa cells.

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