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Evaluation of the Interactions of Cabozantinib with Topoisomerase Enzymes by *in vitro* Enzyme Activity Assays, and its Effects on Cancer Cell Proliferation

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ABSTRACT

The discovery of many drugs in recent years provides a definitive solution in the treatment of various diseases, but today, despite the discovery of many effective anticancer drugs, there are various types of cancer that have limitations in treatment and are still not completely curable. Since most of these limitations are due to cancer cells gaining resistance or compounds only being effective in certain types of cancer cells, the search for more effective anticancer drugs that are also effective in these types of cancer is inevitable. Cabozantinib is in medical use as a highly effective anticancer drug in various types of cancer, such as medullary thyroid cancer and kidney cancer. The anticancer properties of the Cabozantinib compound have attracted more attention in recent years, however, more studies are needed to define the anticancer activities of this compound. In our study, the interactions of Cabozantinib with topoisomerase enzymes, were demonstrated through in vitro enzyme activity tests, and the anti-proliferative effect of Cabozantinib was studied on MCF7, A549 and PC3 cell lines. By analyzing the interactions of the Cabozantinib with topoisomerases, the action mechanisms of the compound at the molecular level was evaluated.

Keywords: Topoisomerase enzymes, Cancer, Cabozantinib, Tyrosine kinase inhibitor, DNA damage.

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Introduction

DNA topoisomerase enzymes play one of the most critical roles in the intact transmission of DNA, our hereditary material, to new generations. How these roles are fulfilled has been explained in detail through extensive research, based on the functions of topoisomerase enzymes in intracellular events such as replication and transcription, and therefore they are primarily targeted in anti-cancer drug development research [1]. Topoisomerase enzymes are divided into two groups according to their ability to create breaks on DNA. In contrast to Topoisomerase I enzymes, which relax DNA by creating single-strand breaks in DNA, Topoisomerase II enzymes simultaneously break double-stranded DNA molecules and allow another double-stranded DNA molecule to pass through them, creating temporary breaks in DNA [2, 3]. Breaks in these DNA strands are reversible unless prevented by an inhibitor, are stabilized according to intracellular mechanisms, and topoisomerase enzymes recombine the strands in a controlled manner. By restricting or inhibiting the activities of these enzymes, uncontrolled and excessive breaks can be created on the DNA. Thus, in light of the knowledge that cancer cells divide rapidly and uncontrollably, aiming to increase the amount of DNA breaks that will disrupt the DNA integrity of these cells is a competent anticancer approach [4–7]. The reasons for targeting topoisomerase I are the remarkable findings that rapidly dividing cancer cells with high topoisomerase I levels are highly sensitive to topoisomerase I inhibitors. Cancer cells become more sensitive to Topoisomerase I-mediated therapies due to overexpression of the topoisomerase I enzyme and associated impairment of DNA repair mechanisms [8, 9].

Clinical Topoisomerase I inhibitors derived from Camptothecin (such as Irinotecan and Topotecan) appear to be highly effective in treating many types of cancer, including colon, ovarian, pancreatic and small cell lung cancers [10-13], whereas Camptothecin analogues Belotecan and Topotecan for sensitive-relapsed small-cell lung cancer (SCLC)[14]. However, some of these cancer types cannot be defeated because they develop drug resistance that reduces the treatment potential of these inhibitors [10-12]. These topoisomerase I-targeting derivatives stabilize topoisomerase I enzymes to form DNA-drug-enzyme complexes and are called enzyme poisons. Because of the fact that the more numerous targets a drug has, the more toxic that drug is, compounds that target too many molecules, such as Camptothecins, create toxicity that must be avoided. However, it is still very reasonable to use less toxic derivatives or combinations of these compounds, since topoisomerase enzymes, whose expression increases in parallel with the rapidly increasing replication of the cancer cell population, will be selectively poisoned by these drugs.

Because of this poisoning mechanism, DNA breaks begin to form early in replication, and although this is a reversible reaction, the DNA breaks reach the amount required to destroy cancerous cells [12, 15]. Morover, Topoisomerase I and Topoisomerase II enzymes are both involved in DNA metabolism, so targeting both of these enzymes can impair resistance resulting from downregulation of the target enzyme and compensatory upregulation of the other topoisomerase enzyme [3, 10, 16].

One of the factors that cause cell death triggered by apoptosis is DNA-damaging compounds that induce many different DNA lesions. It has been stated that DNA damage can be defined as many different DNA modifications that can activate apoptosis, and overexpression of repair proteins involved in DNA repair pathways has a negative effect on cells. The mechanisms by which DNA lesions trigger apoptosis also vary depending on how DNA damage occurs and the cell's reaction to it [17]. Tyrosine kinases regulate DNA damage signaling pathways to orchestrate cell cycle, growth, proliferation, differentiation and survival, while DNA damage response proteins (DDR) alarmed by DNA lesions or chromatin alterations, manipulates the DNA repair system to keep the genome intact [17, 18]. Receptor tyrosine kinases (RTKs) perform functions such as cell proliferation, survival and differentiation, and are targeted for these functions in cancer treatment.

Several tyrosine kinase inhibitors (TKIs) have been approved by the FDA for targeted therapy, and combination therapies with other anticancer drugs or TKIs show promising synergistic effects [19, 20]. Cabozantinib (CBZ) is an orally available and bioavailable small molecule receptor tyrosine kinase (RTK) inhibitor antineoplastic agent. Approved by the US Food and Drug Administration (FDA), CBZ is a drug produced by EXELIS for patients with renal carcinoma, medullary thyroid cancer and kidney cancer [21, 22]. Cabozantinib has been proposed as a compound for the treatment of cases of resistance to drugs that are ABCG2 substrates. As the researchers reported, CBZ sensitized ABCG2-overexpressing cells to the drugs mitoxantrone, SN-38, and topotecan at non-toxic concentrations, thus suggesting Cabozantinib as a compound for treatments of drug resistance that is an ABCG2 substrate [23].

In our study, in order to elucidate one of the molecular mechanisms of how Cabozantinib's anticancer drug properties occur, we investigated the interactions of topoisomerase enzymes with Cabozantinib *in vitro* by performing enzyme activity tests. Whether Cabozantinib has a toxic effect on cancer cells was analyzed by viability assays in different types cancer cell lines for which there is limited data and for the cancer types that have not yet received FDA approval for Cabozantinib treatment.

Methods

All of the compounds used in our experiments were purchased in lyophilized form. Cabozantinib (AdooQ, Irvine, CA, USA), Etoposide (Sigma-Aldrich, St. Louis, MO, USA) were dissolved in 100% Dimethyl Sulfoxide (Sigma-Aldrich, St. Louis, MO, USA). A549 (CCL-185) and PC3 (CRL-1435) cell lines were obtained from ATCC and MCF7 cell line was kindly gifted by Prof Dilek Telci Temeltas, Genetics and Bioengineering Department of Yeditepe University.

Investigation of Topoisomerase-DNA Interactions Enzyme Activity Tests

The reaction products were runned by horizontal electrophoresis (5V/cm) in 1xTAE buffer on a 1% agarose gel. Relaxation assay substrats were runned in the absence of EtBr on agarose gel, while decatenation substrates were runned in the presence of EtBr. Cabozantinib (CBZ) and control compound Etoposide (ETP) were dissolved in 100% DMSO.

Supercoiled DNA Relaxation Assays

Relaxation of supercoiled plasmid DNA was performed in a total of 20 µL volume of buffer (72 mM KCl, 5 mM $MgCl_2$, 5 mM DTT, 5 mM spermidine and 0.1% bovine serum albumin), with 0.5 µg of supercoiled DNA (Takara, Shiga, Japan) and 1 unit of human topoisomerase I enzyme (Topogen, CO, USA). The definition of one unit of topoisomerase enzyme is the amount of enzyme that relaxes 0.5 µg of supercoiled DNA in 30 minutes at 37°C. Compared to the leading bands of supercoiled DNA on the gel, supercoiled DNA, which is less supercoiled by the DNA relaxation activity of the enzyme, appears further back on the gel [24, 25].

Decatenation Assays

In the analysis of decatenation activity reactions, human topoisomerase II (Topogen, CO, USA) enzyme was applied with catenated DNA (kDNA) substrates for 30 minutes at 37 °C in the presence and absence of the test compound [25, 26]. In the assays, 0.2 µg kDNA substrate, and 1 unit of human topoisomerase II enzyme, were interacted in a total of 20 µl final volume in the reaction buffer (50 mM Tris-Cl pH 8.0, 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM ATP, 0.5 mM Dithiothreitol). Enzyme activity was terminated using topoisomerase II stop buffer (5% sarcosyl, 0.0025% bromphenol blue, 25% glycerol).

The Cell Viability Assay

The effect of the CBZ was examined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazoliumbromide (MTT) (Sigma-Aldrich, St. Louis, MO, USA) assay against breast cancer cell line (MCF7), non-small lung cancer cell line (A549) and prostate cancer cell line (PC3). Each cell line was grown and maintained in DMEM (Gibco, Thermo Fisher Scientific, MA, USA) medium containing 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Gibco, Thermo Fisher Scientific, MA, USA), 2mM L-glutamine (Gibco, Thermo Fisher Scientific, MA, USA) and 50 units/mL penicillin-streptomycin (Invitrogen, Thermo Fisher Scientific, MA, USA). 1mg/mL of MTT was applied to the cells. When MTT is applied to living cells, it transforms into

a blue-violet, water-insoluble reduced form, formazan. Determination of viable cell number was calculated by determining the color intensity obtained after dissolving formazan in alcohol by photometric measurements. To investigate the effect of CBZ on MCF7, A549 and PC3 cell viability, CBZ was applied at 5 different concentrations (1.25-20 µM). The cells were seeded at a density of 3500 cells/well a day before treatment with the sample. Subsequently, different concentrations of CBZ were administered to cells. After 72 hours of incubation, formazan formation was determined for each concentration and formazan crystals were dissolved by addition of 150 µL isopropanol. The percentage of viable cells was calculated based on the values acquired by colorimetric methods using Ascent spectrophotometer at 570 nm. Cell group which was not treated with CBZ considered as 100% viable (control group) and its cytotoxic effect calculated as follows: (Compound Abs-Blank Abs)*100/(Control Abs-Blank Abs). Each condition was studied in five replicates.

Results

Supercoiled DNA Relaxation Assays

Before performing the enzyme activity tests, it was analyzed whether CBZ had DNA intercalation activity and it was determined that there was no intercalative formation between DNA and CBZ under the assay conditions. The supercoiled DNA and CBZ bands remained in the lane similar to the band of supercoiled DNA (Figure 1). When CBZ was applied together with supercoiled DNA, the distance of supercoiled DNA running in the gel was the same as the distance of DNA running in the lane where only supercoiled DNA was loaded. The relaxed DNA bands seen in the remaining part of the gel were DNA structures that were physically formed due to the experimental conditions and they ran at a similar distance to each other in the experiment, therefore they were not taken into consideration for examining the effect of DNA intercalation.

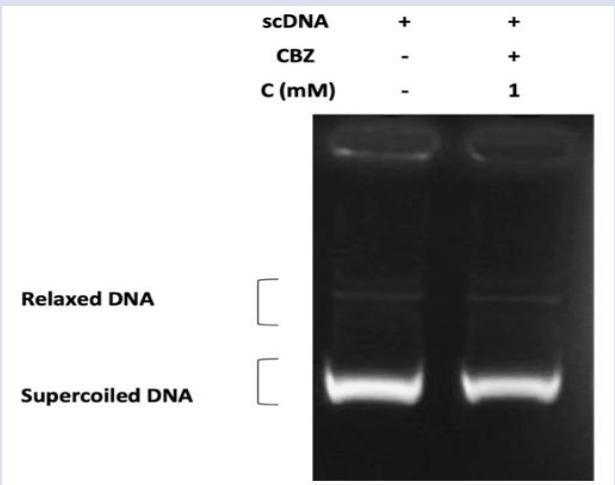

Figure 1. DNA intercalation test. Line 1; 0.5 µg supercoiled DNA, line 2; 0.5 µg supercoiled DNA and 1mM CBZ.

With the interaction of supercoiled DNA and topoisomerase I enzyme, DNA bands relaxed and ran behind in the gel (Figure 2, lane 2). However, due to the inhibition activity that occured when CBZ was added, the DNA bands appeared to run the same distance as the control supercoiled DNA. According to the results of supercoiled DNA relaxation tests, the CBZ compound inhibited the topoisomerase I enzyme in the concentration range of 1- 5 mM (Figure 2, lane 5).

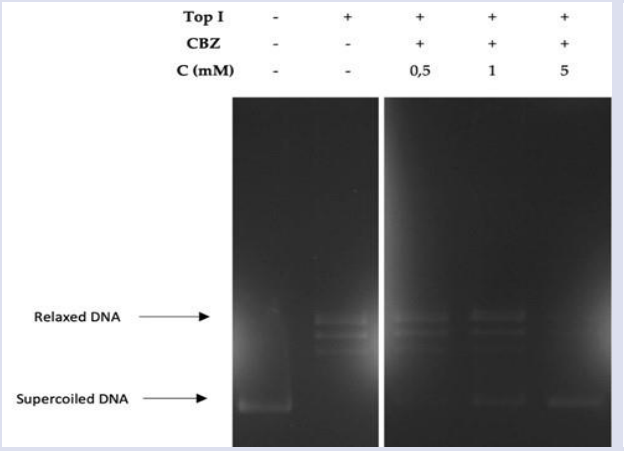

Figure 2. Cabozantinib inhibited topoisomerase I enzyme. Left panel: Line 1; 0.5 µg supercoiled DNA, line 2; 0.5 µg supercoiled DNA and Topoisomerase I enzyme. Right panel: All in the presence of 0.5 µg supercoiled DNA and Topoisomerase I enzyme, line 1; 0.5mM CBZ, line 2; 1 mM CBZ, line 3; 5mM CBZ.

Decatenation Assays

As seen in the gel, catenated DNA was able to come out of the gel by the interaction of Topoisomerase II enzyme (Figure 3, lane 2).

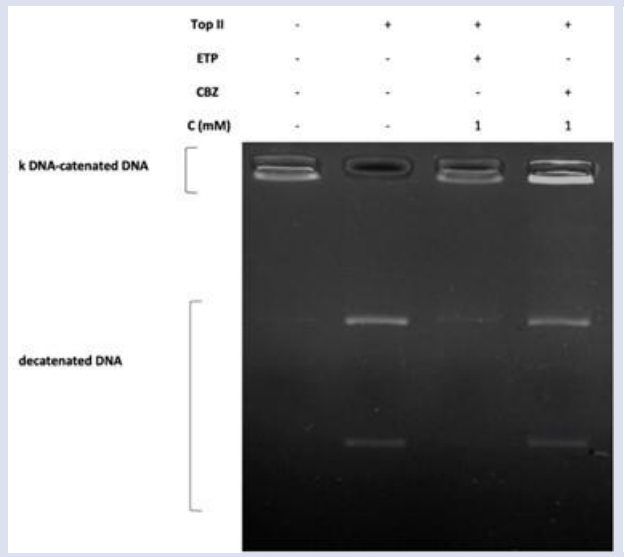

Figure 3. Topoisomerase II decatenation test. Line 1; k-DNA in the absence of topoisomerase II, 2; k-DNA in the presence of topoisomerase II, 3; k-DNA in the presence of topoisomerase II and ETP at 1mM concentration, 4; k-DNA in the presence of topoisomerase II and CBZ at 1mM concentration.

When Etopoiside (ETP) added, catenated DNA (kDNA) could not run in the gel because enzyme activity was lost (Figure 3, lane 3), but CBZ compound at 1mM concentration (Figure 3, line 4) did not show an inhibitory effect similar to ETP (Figure 3, lane 4).

The Cell Viability Assay

The anti-proliferative effect of CBZ was studied on MCF7, A549 and PC3 cell lines. Each cell line grown in 96 well plate, was exposed to CBZ's five different concentrations (1.25-20 µM) for 72 hours. MTT assay results demonstrated that there is no concentration dependent inhibitory effect of CBZ (Figure 4) on cell lines, studied.

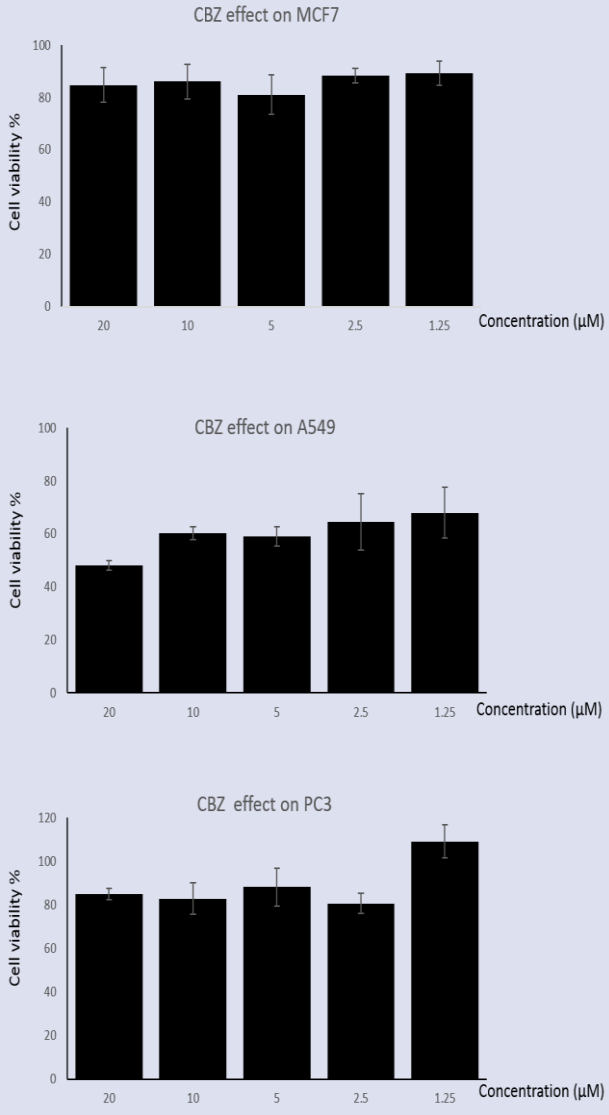

Figure 4. The effect of CBZ on cancer cell viability. MCF7, A549 and PC3 cell lines were treated with CBZ at indicated concentrations for 72 hours. MTT test was performed to determine cytotoxic effect of CBZ. For each panel, the group of cells which was not administered to CBZ was considered as exhibiting 100% proliferation. Each condition studied in 5 replicates. Mean ±std.

Discussion

By preferring anticancer molecules that cause breaks in DNA to rapidly proliferating cancer cells, many intracellular pathways can be blocked and rapid destruction of cancer cells can occur [27, 28]. With the mentioned inhibitory activity, the cell's ability to relieve the stress provided by topoisomerases is prevented, and a sad drama occurs in the cancerous cell, in which the DNA strands are torn into pieces and the cell is dragged into apoptosis [29, 30]. Protein kinases have important roles in cell signaling and help destroy cancer cells through apoptosis. The majority of cancers are known to rapidly develop resistance to kinase inhibitors administered alone; therefore, a molecule that interacts with more than one kinase appears critical to be a successful anticancer drug. Depending on the signal transmission received in the cell, they change the enzyme activity in the target protein and its interactions with other proteins [21, 22]. In terms of frequency and appropriateness of use in clinical practice, most of the approved and recommended TKIs have not yet achieved significant clinical success. Among the highlights, it is noteworthy that combination treatments provide successful results. It is suggested that checkpoint inhibitors and epigenetic modifiers combined with TKI may show significant effectiveness in many types of cancer, including lymphoma cells [31, 32]. For the targeted treatment of unresectable HCC, Lenvatinib, Cabozantinib, Ramucirumab and Regorafenib have been approved [33].

Cabozantinib is a multi-targeted small-molecule TKI, which targets VEGFR, MET and AXL and it has gained importance that Cabozantinib is effective after Sorafenib administration in the treatment of HCC (Cabometyx® brand). The effectiveness of this compound under another brand, for the treatment of "unresectable locally advanced" or "metastatic Medullary thyroid cancer (MTC)", has also come to the fore (Cometriq® brand). Because the Cabometyx® is formed as a tablet and Cometriq® formed as a capsule, it has been reported that their formulation can not be fully equal indeed [20, 32]. A phase II study expected to reveal the efficacy of Cabozantinib in the treatment of patients with incurable, refractory, germ cell tumors, including ovarian germ cell tumors, have started in May 2021 (NCT04876456). However, in cases where the response to immunotherapy in ovarian cancer is unfortunately low, the effectiveness of Cabozantinib in this type of cancer has become a matter of possibility [32]. According to a phase II study reported in recent years, the effectiveness of Cabozantinib in the second- and third-line treatments of clear cell ovarian, fallopian tube or primary peritoneal carcinoma was limited to a minimal level [34]. In addition to all these promising results, CBZ has also been proven to have an effect on other types of cancer cells. When administered in combination with Erlotinib and Cabozantinib, antitumor effects against non-small cell lung cancer cells have been demonstrated in both *in vitro* and *in vivo* models [35].

In our study, the interactions of this compound with topoisomerase I and topoisomerase II enzymes was investigated by subjecting it to *in vitro* topoisomerase enzyme activity assays and cell viability tests. According to the results, Cabozantinib did not have a significant concentration-dependent inhibitory effect on MCF7, A549 and PC3 cell lines. However, Cabozantinib inhibits human topoisomerase I enzyme but not human topoisomerase II enzyme *in vitro* conditions, providing a *bona fide* information about the pathways through which the compound acts. Further analyzes are needed to answer questions such as whether this chemical will give successful results when applied alone to different cancer cell types and whether its effectiveness will increase with combination applications.

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Conflicts of Interest

The authors declare no conflict of interest.

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