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Expression of hCA IX isoenzyme by using sumo fusion partner and examining the effects of antitumor drugs

Sumo füzyon partneri kullanarak hCA IX izoenziminin ekspresyonu ve antitümör ilaçların etkilerinin incelenmesi

Abstract: Objective: In this study, investigating the effects of inhibition of the enzyme activity of some antitumor drugs and the Cancer-Related Human Carbonic Anhydrase IX (hCA IX) isoenzyme expressing as a SUMO fusion protein in an *Escherichia coli* expression system were aimed.

Methods: hCA IX isoenzyme was expressed using SUMO fusion technology. The fusion protein was expressed in a totally soluble form and the expression was verified by SDS-PAGE analysis. Affinity chromatography was used in the purification processes. The effects of certain antitumor drugs on enzyme activity were investigated *in vitro* conditions by using esterase activity. IC_{50} values of drugs showing the inhibitory effect were calculated. Inhibition types and K_i values for antitumor drugs, which inhibit the enzyme, were determined by separately plotting Lineweaver-Burk plots.

Results: The molecular weight of the fusion protein was approximately 85kDa. The optimal induction concentration of IPTG and the growth temperature were found to be 1.0mM and 30°C. The fusion protein was purified at approximately 3.07-fold with a yield of 92.58%, and a specific activity of 43707EU/mg proteins by nickel nitrilo-triacetic acid resin chromatography.

Conclusion: Our work is extremely important because CA IX plays a clinical role as a biomarker in cancer diagnosis and the use of specific inhibitors of the CA IX enzyme will be useful in the fight against cancer. *In vitro* inhibition studies on the recombinant hCA IX enzyme can shed light on the development of anticancer drugs for cancers overexpressing CA IX.

Keywords: Cloning, expression, cancer, carbonic anhydrase IX, inhibition

Özet: Amaç: Bu çalışmada kanser ilişkili İnsan Karbonik Anhidraz IX (hCA IX) izoenziminin *Escherichia coli* ekspresyon sistemi içinde bir SUMO füzyon proteini olarak ekspre edilmesi ve bazı antitümör ilaçların enzim aktivitesi üzerine inhibisyon etkilerinin incelenmesi amaçlanmıştır.

Metod: hCA IX izoenzimi SUMO füzyon teknolojisi kullanılarak ekspre edildi. Füzyon protein tümüyle çözünür bir formda ekspre edildi ve ekspresyon SDS-PAGE analizi ile doğrulandı. Saflaştırma işlemlerinde afinite kromatografisi kullanıldı. Enzim aktivitesi üzerine bazı antitümör ilaçların etkileri *in vitro* şartlarda esteraz aktivitesi metodu kullanılarak incelendi. İnhibitör etkisi gösteren

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ilaçların IC_{50} değerleri hesaplandı. Enzimi ihhibe eden antitümör ilaçlar için K_i değerleri ve inhibisyon tipleri Lineweaver-Burk grafikleri ayrı ayrı çizilerek belirlendi.

Bulgular: Füzyon proteinin molekül ağırlığı yaklaşık olarak 85 kDa'du. Optimal IPTG indükleme konsantrasyonu ve büyüme sıcaklığı 1,0 mM ve 30°C olarak bulundu. Füzyon protein nikel-nitrilotriasetik asit (Ni–NTA) afinite kromatografisi kullanılarak yaklaşık olarak 43707 EUxmg-1 spesifik aktiviteyle; %92,58 verimle; 3,07 saflaştırma katsayısıyla saflaştırıldı.

Sonuç: CA IX enzimi, kanser tanısında belirteç olarak klinik rol oynadığından ve CA IX enziminin spesifik inhibitörlerinin kullanımı kansere karşı mücadelede yararlı olacağından dolayı çalışmamız önem arzetmektedir. Rekombinant hCA IX enzimi üzerine yapılacak olan *in vitro* inhibisyon çalışmaları CA IX'un aşırı ekspresyon gösterdiği kanserler için antikanser ilaçlarının geliştirilmesine ışık tutabilir.

Anahtar Kelimeler: Klonlama, ekspresyon, kanser, karbonik anhidraz IX, inhibisyon

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Introduction

Carbonic anhydrase IX (CA IX) is a transmembrane glycoprotein, a member of a family of zinc metalloenzymes that reversibly converts carbon dioxide and water to carbonic acid contributing to an acid-base balance. CA IX plays a relevant role in pH regulation and its expression reduces the pericellular pH, facilitating breakdown of the extracellular matrix [1]. CA IX transmembrane protein has been linked to carcinogenesis. It has been suggested that this membrane-bound CA IX isoenzyme participates in cancer cell invasion, which is facilitated by an acidic tumour cell environment. In contrast to the other isoenzymes, it has been implicated in the regulation of cell proliferation, adhesion, and malignant cell invasion. While most CA isoforms are uniformly expressed in normal differentiated cells, CA IX is predominant in cancer cells [2]. CA IX expression has been correlated with a clinical outcome in several types of human cancers. In human cancers, CA IX contributes to maintain intracellular and extracellular pH under hypoxic conditions, but also influences the regulation of cell proliferation and tumour progression. CA IX was indicated as an independent prognostic marker in non-small cell lung carcinoma [3,4]. Published clinical studies provide conflicting data regarding the prognostic significance of CA IX overexpression as an endogenous marker of tumour hypoxia and its comparability with other methods of hypoxia detection [5]. Recently, a convincing body of evidence has accumulated suggesting that the overexpression of CA IX in certain types of cancers contributes to the acidification of the extracellular matrix, which in turn promotes the growth and metastasis of the tumour. These observations have made CA IX an attractive drug target for the selective treatment of certain types of cancers [6].

Fusion systems are known to increase the purification and expression of soluble form of recombinant proteins. Small ubiquitin-related modifier (SUMO) fusion proteins purification processes are also simplified. 6xHis-tagged SUMO-fusions facilitate rapid purification of proteins on a large scale. SUMO fusions increase solubility of target proteins in *E. coli* [7–9].

The Docetaxel drug is used for the prevention and treatment of gastric cancer, breast cancer, non-small cell lung cancer, ovarian cancer, head-neck cancer and prostate cancer. Docetaxel is an anti-neoplastic drug. The drug is used in cancer treatment, either alone or in combination with other drugs. Paclitaxel is an anti-neoplastic drug that is frequently used nowadays in the treatment

Table 1: Summary of purification procedure for the SUMO/hCA IX fusion protein and purified hCA IX.

Purification steps	Activity (U/ml)	Protein (mg/ml)	Total volume (ml)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification fold
Homogenate	5067	0.356	12	60804	4.272	14233	100	1
Sumo–hCA IX after Ni-NTA Affinity Chromatography	8042	0.184	7	56294	1.288	43707	92.58	3.07
hCA IX after Ni–NTA Affinity Chromatography	13067	0.045	4	52268	0.180	290378	85.96	20.4

of advanced non-small cell lung cancer, breast cancer and ovarian cancer. Irinotecan HCI is an anti-neoplastic drug that is used nowadays in the treatment of advanced ovarian cancer and resistant colon cancer. Etoposide is an anti-neoplastic drug that is effectively used in the treatment of advanced non-small cell lung, testes and ovarian cancers [10].

In this study, we report the application of SUMO fusion technology. We describe in detail the cloning of the human carbonic anhydrase IX (hCA IX) gene into a petSUMO vector, the expression of SUMO-hCA IX in a soluble form, purification and the biological assay of recombinant hCA IX. We investigated the effects of inhibition of certain antitumor drugs on the recombinant hCA IX activity of *in vitro* conditions using the method of esterase activity.

The importance of this study, which was made for elucidating the mechanism of inhibition of CA IX isoenzyme, is clear. *In vitro* inhibition studies on the recombinant hCA IX enzyme can shed light on the development of potential anticancer drugs for cancers overexpressing CA IX.

Materials and Methods

Chemicals

Escherichia coli (JM109) were used for subcloning and plasmid amplification. *Escherichia coli* BL21 (DE3) were used as the expression host. The strains bacteria were stored at -80°C until usage. Champion™ pET SUMO Protein Expression System and SuperScript® III First-Strand was purchased from Invitrogen, USA. GenElute™ Plasmid Miniprep Kit, Lysozyme from chicken egg white, dNTP Mix, Taq DNA Polymerase, Ampicillin, Kanamycin sulphate, Albumin from bovine serum (BSA), GenElute™ Gel Extraction Kit and *N*,*N*,*N*′,*N*′-Tetramethylethylenediamine was purchased from Sigma-Aldrich, USA. Human Pancreas Poly A+ RNA was purchased from Clontech, USA.

Cloning into pGEM-T Easy Vector

hCA IX gene was amplified by using specific primers (Fw: 5'- ATG GCT CCC CTG TGC CCC A -3' and Rev: 5'- GGC TCC AGT CTC GGC TAC C -3') with PCR from the human pancreas cDNA library according to sequences reported in the NCBI data bank [11]. The PCR fragments were separated by 1.0% agarose gel electrophoresis, purified with a DNA gel extraction kit. The resulting PCR product was ligated into the pGEM-T Easy vector. The ligation mixture was transformed into competent *E. coli* (JM109) cells. The transformed cell culture was plated onto agar plates containing ampicillin, IPTG (isopropyl-beta-D-thiogalactopyranoside) and X-GAL (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). The plates were sealed and incubated overnight at 37°C. Blue and white (recombinant) colonies occurred on petri. The plasmid obtained from white (recombinant) colonies was confirmed by DNA sequencing and named pGEM-T Easy-hCA IX.

Determination of DNA Sequence and Analysis

Sequence analysis of pGEM-T Easy/hCA IX plasmid was performed by the company Applied Biosystems and DNA sequence of hCA IX gene was confirmed.

Cloning into petSUMO Expression Vector

hCA IX gene was amplified by using specific primers (Fw: 5'- ATG GCT CCC CTG TGC CCC A -3' and Rev: 5'- GGC TCC AGT CTC GGC TAC C -3') with PCR from pGEM-T Easy/hCA IX plasmid. The PCR fragments were separated by 1.0% agarose gel electrophoresis, purified with a DNA gel extraction kit. The resulting PCR product was ligated into the petSUMO expression vector. The ligation mixture was transformed into competent *E. coli* BL21 (DE3) cells. The transformed cell culture was plated onto agar plates containing kanamycin. The plates were sealed and incubated overnight at 37°C. White colonies occurred on petri.

Expression of SUMO Fusion Protein

A single colony was selected. The cells were cultured overnight in a 8 ml sterilised Luria–Bertani (LB) medium containing 50 μg ml-1 kanamycin at 37°C shaken at 130 rpm, diluted 1:100 with fresh pre-warmed LB medium containing 50 μg ml^{-1} kanamycin and incubated at 37°C shaken at 200 rpm. When OD_{600} reached a value of approximatelyt 0.8, the expression of the petSUMO-hCA IX recombinant protein was induced by adding 1mM IPTG and 400 μM zinc sulfate for an additional 8 hours at 30°C shaken at 260 rpm. The cells were harvested by centrifugation at 6.000 g for 15 minutes at 4°C and immediately until further processing.

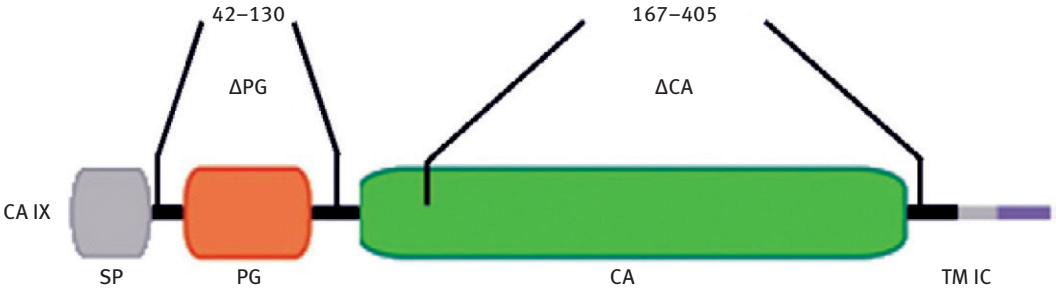


Figure 1: Domain organization of the CA IX protein: SP=signal peptide; PG=proteoglycan-like domain, CA=catalytic domain; TM=transmembrane segment; IC=intracellular, cytosolic tail. Ex vivo experiments with deletion constructs (ΔPG or ΔCA) have been performed for establishing the role of the protein in tumor acidification processes, see discussion in the text.

Purification of SUMO Fusion Protein

The cells that frozen at -20°C were taken to room temperature and allowed to stand until dissolved. The cells are lysed using liquid nitrogen with help of a mortar and pestle. The cells resuspended in precooled lysis buffer (BLB, pH=8.0). The homogenate were centrifuged at 17.000 g for 20 minutes at 4°C. The resultant supernatant was loaded onto a nickel nitrilo-triacetic acid (Ni–NTA) resin affinity chromatography column equilibrated with binding buffer (20 mM sodium phosphate, 500 mM sodium chloride, pH 8.0). Nonspecifically bound proteins were removed by washing with washing buffer. After extensive washing with washing buffer (20 mM sodium phosphate, 500 mM sodium chloride, and 20 mM imidazole, pH 8.0), the fusion protein was eluted with five column volumes of elution buffer (20 mM sodium phosphate, 500 mM sodium chloride, and 250 mM imidazole, pH 8.0). The peak fractions containing the fusion protein were pooled and dialyzed overnight at 4°C against phosphate buffered saline (PBS, pH 7.4).

SDS–PAGE and Protein Determination

After the purification steps, SDS polyacrylamide gel electrophoresis was performed in order to verify enzyme purity. It was carried out in 10% and 3% acrylamide for the running and the stacking gel, respectively, containing 0.1% SDS according to Laemmli procedure [12]. A 20 µg sample was applied to the electrophoresis medium. Gels were stained for 90 minutes in 0.1% Coommassie Brilliant Blue R-250 in 50% methanol and 10% acetic acid, then destained with several changes in the same solvent without a dye. Protein concentration was determined spectrophotometrically at 595 nm according to the Bradford method using the bovine serum albumin as the standard [13].

Cleavage of SUMO Fusion Protein and Purification of hCA IX

The dialyzed fusion protein was reacted with SUMO protease (1 U) per 50 µg fusion protein at 30°C for 1 hour. Since both SUMO and SUMO protease had 6xHis tags, but hCA IX did not, the cleaved SUMO fusion samples could be reapplied to the nickel column to obtain the purified hCA IX by subtracting the 6xHis-tagged proteins. Briefly, after the SUMO fusions were cleaved by the SUMO protease, the sample was loaded onto a nickel column with Ni–NTA resin. Most of the hCA IX without 6xHis tags was eluted in the flow through (unbound) fractions, and the rest was recovered by washing the resin with binding buffer. The eluted and washed proteins appearing in fractions with high-UV values at A_{280} were pooled as the final purified sample. The purified proteins were checked on SDS–PAGE and the samples were stored at -80°C for activity assay.

Esterase activity assay

The esterase activity was assayed by following the change in absorbance of 4-nitrophenylacetate (NPA) to 4-nitrophenol at 348 nm over a period of 3 minutes at 25°C using a spectrophotometer (BECKMAN COULTER UV-VIS) according to the method described by Verpoorte *et al.* [14]. The enzymatic reaction, in a total volume of 3.0 mL, contained 1.4 mL 0.05 M Tris-sulfate buffer (pH 7.4), 1.0 mL 3 mM 4-nitrophenylacetate, 0.5 mL water and 0.1 mL enzyme solution. A reference measurement was obtained by preparing the same cuvette without enzyme solution. This activity is measured in terms of the activity unit (U) which is defined as the amount which will catalyse the transformation of 1 micromole of the substrate per minute under standard conditions.

Kinetic studies

The K_m and V_{max} of the enzyme values were calculated from a Lineweaver-Burk [15] plot, at room temperature and optimum pH, in 1 mL reaction mixtures as described above. The experiment was performed at 5 different cuvette concentrations of p-nitrophenyl acetate (0.3, 0.45, 0.6, 0.75, and 0.9 mM) [16,17].

The *in vitro* inhibition effect of some antitumor drugs on the recombinant hCA IX activity

In this study, the inhibitory effects of certain antitumor drugs were investigated at different inhibitor concentrations. hCA IX activity was measured in the presence of different drug concentrations. For paclitaxel, docetaxel, irinotecan HCL and etoposide, inhibition studies were performed by using esterase activity. The control activity in the absence of inhibitor was taken as 100%. For each drug, activity (%) vs. inhibitor concentration graphs were drawn. In Table 2, the IC_{50} values were reported at five different inhibitor concentrations.

In order to determine K_i constants in the media with inhibitor, the substrate (NPA) concentrations were 0.3, 0.45, 0.6, 0.75, and 0.9 mM. Inhibitor solutions were added to the reaction medium, resulting in three different fixed concentrations of inhibitors in 1 mL of the total reaction volume. Lineweaver-Burk graphs were drawn by using 1/V vs. 1/[S] values and K_i constant were calculated from these graphs. In Table 2, the K_i values were reported at three different inhibitors and five different substrate concentrations by using the esterase activity method [15].

Results and Discussion

The expression and purification of membrane proteins at high levels are an arduous task because the membrane proteins are strongly hydrophobic and they fold incorrectly and may form aggregate, leading to either rapid degradation or accumulation as inclusion bodies.

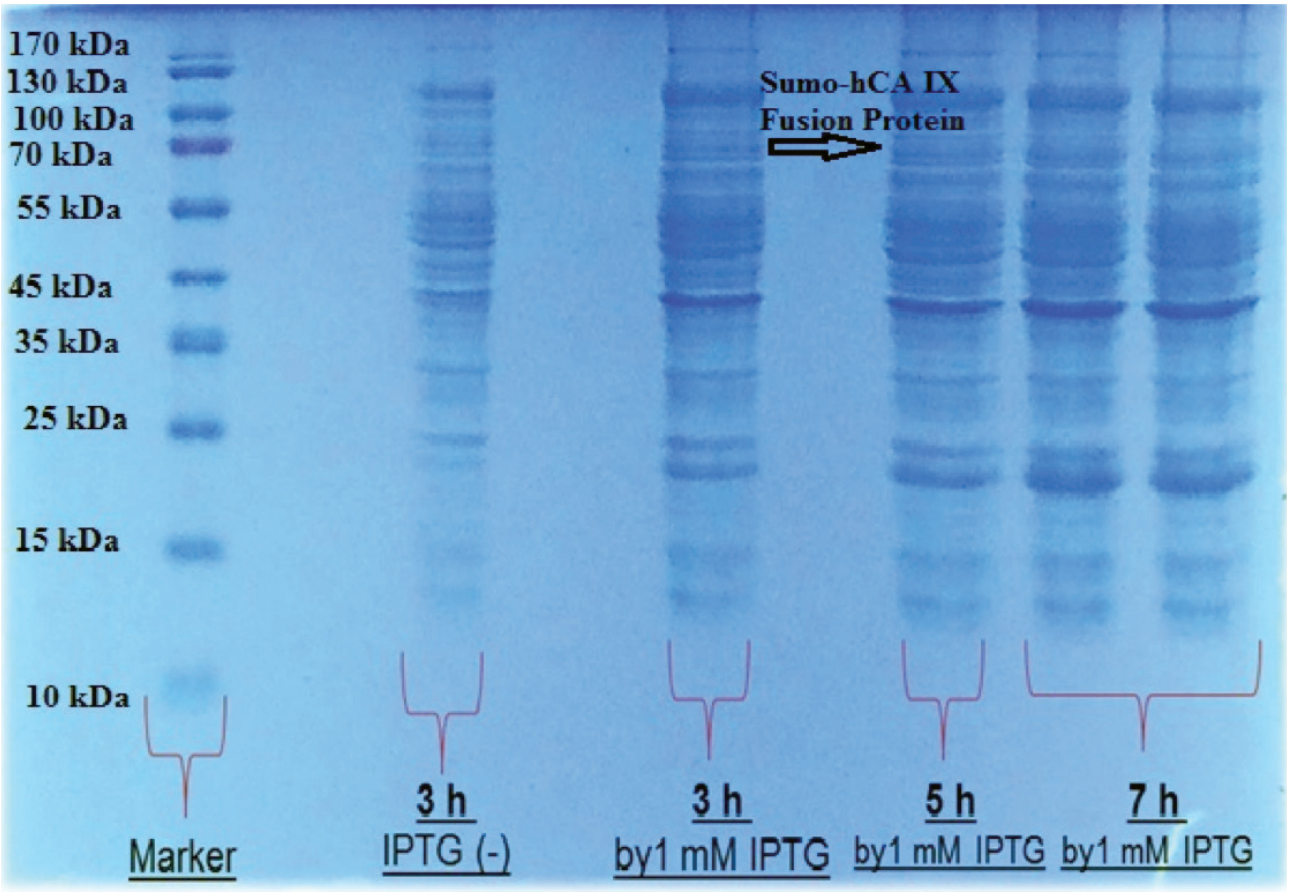


Figure 2: Expression of Sumo-hCA IX fusion protein by using IPTG. Marker (170 kDa), (130 kDa), (100 kDa), (70 kDa), (55 kDa), (45 kDa), (35 kDa), (25 kDa), (15 kDa) and (10 kDa).

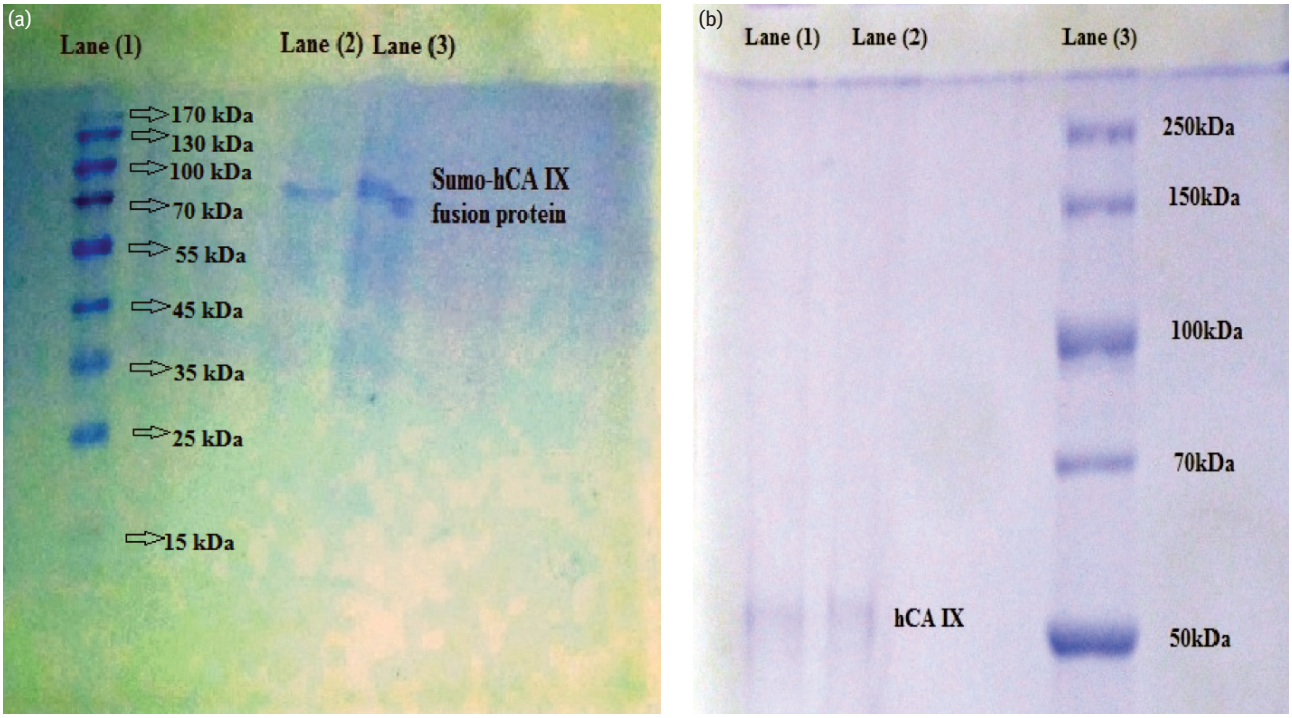


Figure 3: (a) SDS-PAGE analysis of purified Sumo-hCA IX fusion protein. Lane (1) standard proteins (170 kDa), (130 kDa), (100 kDa), (70 kDa), (55 kDa), (45 kDa), (35 kDa), (25 kDa), (15 kDa) and (10 kDa). Lane (2-3) Sumo-hCA IX fusion protein. (b) SDS-PAGE analysis of purified hCA IX enzyme. Lane (1-2). hCA IX enzyme. Lane (3) standard proteins (250 kDa), (150 kDa), (100 kDa), (70 kDa) and (50 kDa).

However, if the membrane proteins are expressed as a fusion protein, this problem is alleviated to some extent [18]. In this study, we have eliminated this problem by using fusion technology.

The hCA IX gene is encoded as a 459 amino acid (aa) residues protein, the 414 aa N-terminal extracellular part linked through a 20 aa polypeptide hydrophobic transmembrane region (TM) and a 25 aa residue C-terminal intracellular tail (IC). Therefore the extracellular part is composed of a signal peptide (37 aa long), the proteoglycan (PG) domain, consisting of 59 aa polypeptides (with similarity to the keratan sulphate-binding domain of a large proteoglycan aggrecan) and a 257 aa long polypeptide, which possesses a large homology to the mammalian CA domain [19,20], as shown in Figure 1.

In this study, the hCA IX gene was amplified from the human pancreas cDNA library by using specific primers with PCR. The PCR fragments were loaded on 1% agarose gel, stained with EtBr and visualised using the gel documentation system. The expected size of the amplified band was 1377 bp. The hCA IX gene was purified from agarose gel and cloned into the pGEM T Easy cloning vector. The transformed cell culture was plated onto agar plates containing ampicillin, IPTG and X-GAL. The plates were sealed and incubated overnight at 37°C. White colonies represent positive cloning and they were selected for subsequent verification. The pGEM T Easy/hCA IX plasmid was purified from the transformed cell culture by using the GenElute™ Plasmid Miniprep Kit. Sequence analysis of pGEM T Easy/hCA IX plasmid was performed by the company Applied Biosystems. The DNA sequence of the hCA IX gene was confirmed.

The hCA IX gene was amplified from purified pGEM T Easy/hCA IX plasmid by using specific primers with PCR. The PCR fragments were loaded on 1% agarose gel, stained with EtBr and visualised using the gel documentation system. Specific band matches the corresponding size of the hCA IX. The hCA IX gene was purified from agarose gel and cloned into the pet SUMO expression vector. The transformed cell culture was plated onto agar plates containing kanamycin. The plates were sealed and incubated overnight at 37°C. The Cancer-Related hCA IX isoenzyme was then successfully expressed as a SUMO fusion protein in an *E. coli* expression system. The fusion protein was expressed in totally soluble form and expression was verified with SDS–PAGE analysis (Figure 2). The optimal induction concentration of IPTG and growth temperature was found to be 1.0 mM and 30°C.

In this study the fusion protein was purified at approximately 3.07-fold with a yield of 92.58%, and a specific activity of 43707 U/mg proteins by Ni–NTA resin chromatography. After the SUMO/hCA IX fusion protein was

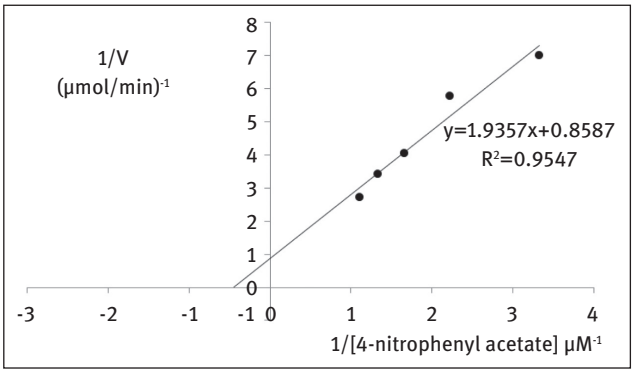


Figure 4: Lineweaver-Burk graph for 5 different substrate concentrations.

cleaved by the SUMO protease, the cleaved sample was reapplied to a Ni–NTA column. The hCA IX was purified at approximately 20.4-fold with a yield of 85.96%, and a specific activity of 290378 U/mg proteins by the same column (Table 1).

The purity and molecular weight of the fusion protein and the hCA IX enzyme were determined by using the SDS-PAGE method (Figure 3a, b). The MW of the purified enzymes were calculated as ~85 kDa for the fusion protein and ~50.47 kDa for hCA IX from Log MW vs. R_f graphs (R^2=09529), respectively.

Kinetic constants, K_m and V_{max} of the enzyme for hydrolysis of p-nitrophenyl acetate were determined as 2.25 mM and 0.858 µmol minute^{-1} based on Lineweaver-Burk plots (Figure 4), respectively.

For paclitaxel, docetaxel, irinotecan HCL and etoposide, which is frequently used nowadays for chemotherapy, inhibition studies were performed by using esterase activity. IC_{50} values were reported at five different inhibitor concentrations (Table 2). When the IC_{50} values of antitumor drugs were compared to each other, the rank of drugs are as follows from the smallest to the greatest: Irinotecan HCL<Paclitaxel<Docetaxel<Etoposide. Numerical data was determined as 0.40 mM<0.79 mM<2.71 mM<5.84 mM in the same order (Figure 5a–d). According to these results, Irinotecan HCL antitumor drugs, which are frequently used in the treatment of advanced ovarian cancer and resistant colon cancer, achieved the highest inhibitory effect in antitumor drugs. Etoposide demonstrated the lowest inhibitory effect in antitumor drugs. As can

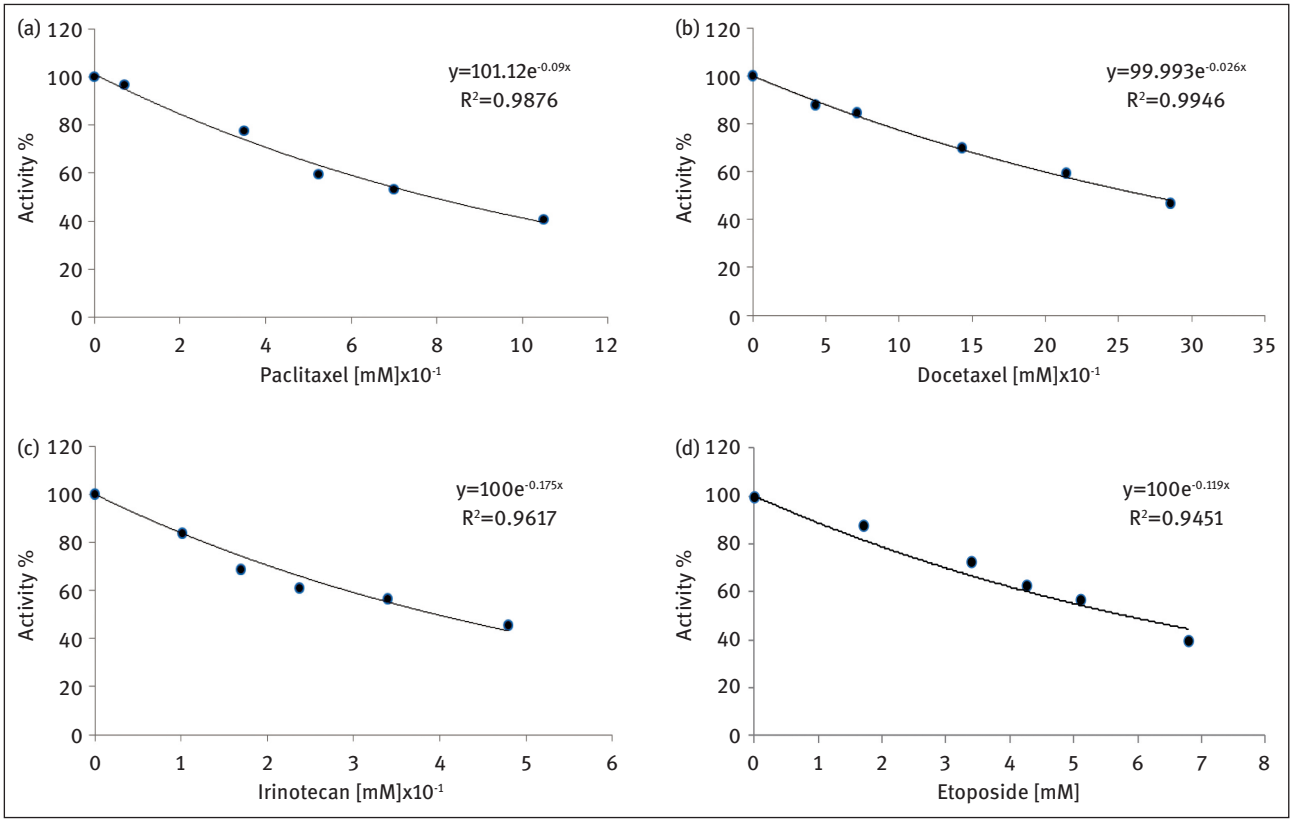


Figure 5: (a) Activity%(0.64U/ml)-[Paclitaxel] graph obtained in five different Paclitaxel concentrations for the activity of hCA IX isoenzyme.
(b) Activity%(0.64U/ml)-[Docetaxel] graph obtained in five different Docetaxel concentrations for the activity of hCA IX isoenzyme.
(c) Activity%(0.64U/ml)-[Irinotecan HCL] graph obtained in five different Irinotecan HCL concentrations for the activity of hCA IX isoenzyme.
(d) Activity%(0.64U/ml)-[Etoposide] graph obtained in five different Etoposide concentrations for the activity of hCA IX isoenzyme.

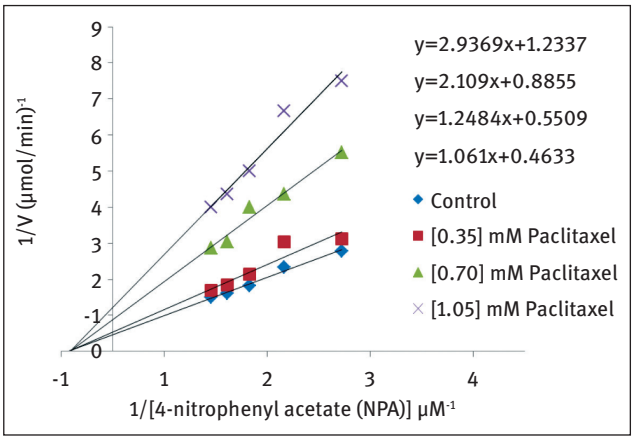


Figure 6: Lineveawer-Burk graph which shows the effect of Paclitaxel antitumor drug on hCA IX isoenzyme.

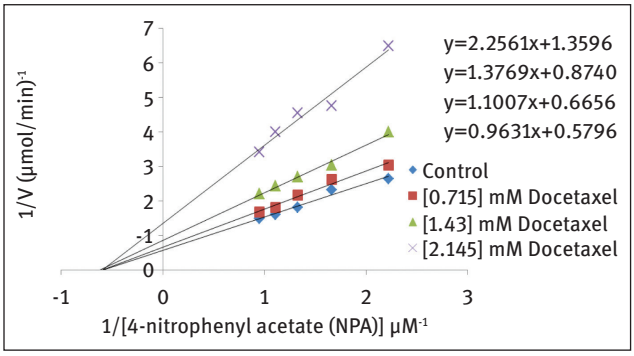


Figure 7: Lineveawer-Burk graph which shows the effect of Docetaxel antitumor drug on hCA IX isoenzyme.

be seen from the results, these antitumor drugs show the inhibitory effect on the enzyme activity, even at very small concentrations.

Lineweaver-Burk graphs were drawn by using 1/V vs. 1/[S] values. K_i values were determined at three different inhibitors and five different substrate concentrations (Table 2). Depending on the difficulty of procurement of medicaments only Paclitaxel and Docetaxel antitumor drugs studies were performed. In the result of our study, the K_i value of Paclitaxel antitumor drug, which is frequently used nowadays in the treatment of advanced non-small cell lung cancer, breast cancer and ovarian cancer, was 1.65 mM (Figure 6) and the K_i value of the Docetaxel antitumor drug, which is used in the prevention and treatment of gastric cancer and head-neck cancer, prostate cancer, was 1.86 mM (Figure 7). When we determine the types of inhibition by looking at the Lineweaver-Burk graphs, it was identified that both of the drugs exhibited a non-competitive inhibition (Table 2). It has been identified that the substrate is not in competition with inhibitor for an active site. The inhibitors has showed inhibition effects without binding to an active site. Our study obtained these findings:

- In more detailed elucidation of the mechanism of inhibition of CA IX isoenzyme,
- In the development of specific, more powerful and effective inhibitors against to CA IX,
- In the explanation of the connection between the spread of cancer with activity of the CA IX isoenzyme more clearly, which is vital in the metabolism,
- In the pharmacological applications and the design of new drugs for the treatment of cancer,

will shed light on the studies that will be carried out and in this context, it will provide a significant contribution to the literature.

In conclusion, our work is extremely important because CA IX plays a clinical role as a biomarker in cancer diagnosis and the use of specific inhibitors of the CA IX enzyme will be useful in the fight against cancer. *In vitro* inhibition studies on the recombinant hCA IX enzyme can shed light on the development of potential anticancer drugs for cancers overexpressing CA IX.

Acknowledgements: This work has been funded with the support of project number BAP 2011/95 of Atatürk University.

Table 2: Inhibition types, IC_{50} and Ki values for antitumor drugs, which inhibit the enzyme.

Antitumor Drugs		IC_{50} (mM)
Irinotecan HCL		0.40
Paclitaxel		0.79
Docetaxel		2.71
Etoposide		5.84
Antitumor Drugs	**Average K_i (mM)**	**Inhibition Types**
Paclitaxel	1.65	Noncompetitive
Docetaxel	1.86	Noncompetitive

Conflict of Interest: The authors have no conflict of interest.

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