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Inhibition Properties of Some Pyrimidine Derivatives as Anticancer Agents on Glutathione S-Transferase

Glutatyon S-Transferaz Enzimi Üzerine Antikanser Ajanlar Olarak Bazı Pirimidin Türevlerinin İnhibisyon Özellikleri

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Abstract

Objective: Glutathione S-transferase (GST; EC 2.5.1.18), which is a member of the family of transferase enzymes, is an enzyme that has a very important role in maintaining the internal and external balance of the body by catalyzing the first stage of the formation reactions mercapturic acid, the end product of detoxification metabolism. In this study, it was aimed to investigate the *in vitro* inhibition effect of pyrimidine derivatives on the GST enzyme.

Materials and Methods: The GST enzyme was purified by affinity chromatography technique. It was determined that pyrimidine derivatives strongly inhibited the GST enzyme at μM level with K_I values ranging from 0.047 ± 0.0015 to 0.272 ± 0.1764 μM. IC_{50} and K_I values were calculated for the derivative compounds.

Results: All of the compounds whose effect was examined showed a noncompetitive inhibition effect on the GST enzyme. In addition, 4-amino-2-chloropyrimidine showed the most effective inhibitory effect with non-competitive inhibition type and the lowest K_I value (0.047 ± 0.0015 μM).

Conclusion: The findings obtained from this study make many contributions to the literature. It is thought that these pyrimidine derivative compounds, the effect of which was investigated in the study and showing an inhibitory effect on the GST enzyme, may shed light on further biological studies.

Key Words: Pyrimidine; Enzyme; Inhibition.

Özet

Amaç: Transferaz enzim ailesinden olan Glutatyon S-Tranferaz (GST; EC 2.5.1.18), detoksifikasyon metabolizması son ürünü olan merkapturik asit oluşum reaksiyonlarının ilk basamağını katalizleyerek, vücudun iç ve dış dengesini korumada çok önemli rolü olan bir enzimdir. Bu çalışmada, pirimidin türevlerinin GST enzimi üzerine *in vitro* inhibisyon etkisinin incelenmesi amaçlanmıştır.

Gereç ve Yöntem: Afinite kromatografi tekniği ile GST enzimi insan eritrositlerinden saflaştırıldı. Pirimidin türevlerinin GST enzimini K_I değerlerinin 0.047 ± 0.0015 ile 0.272 ± 0.1764 arasında μM seviyede güçlü bir şekilde inhibe ettiği belirlendi. Türev bileşikler için IC_{50} ve K_I değerleri hesaplandı.

Bulgular: Etkisi incelenen bileşiklerin tümü GST enzimi üzerine yarışmasız inhibisyon etkisi gösterdi. Ayrıca, 4-amino-2-kloropirimidin yarışmasız inhibisyon tipi ve en düşük K_I değeri ile (0.047 ± 0.0015 μM) en etkili inhibitör etkisi göstermiştir.

Sonuç: Bu çalışmadan elde edilen bulgular, literatüre pek çok katkı sağlamaktadır. Çalışmada etkisi araştırılan ve GST enzimi üzerine inhibisyon etkisi gösteren bu pirimidin türevi bileşiklerin daha ileri biyolojik çalışmalara ışık tutabileceği düşünülmektedir.

Anahtar Kelimeler: Pirimidin; Enzim; İnhibisyon.

Objective

With the damage to the cells, there is an increase in the production of reactive oxygen species (ROS) produced by aerobic cells. Physiological levels of ROS required for cell survival are also mediators for intracellular signaling pathways. On the contrary, excessive amounts of ROS cause cell damage and even cell death (1). For years, it has been thought that oxidative stress is effective in cancer formation and development and therefore cancer can be prevented by antioxidant therapy (2). Glutathione, a tripeptide, consists of cysteine, glycine and glutamate amino acids. Glutathione has two peptide bonds, two carboxyl groups, an amino group and a thiol group (3). There are two types of glutathione, reduced (GSH) and oxidized (GSSG). The excess of hydrophilic groups and the small molecular mass provide an increase in the solubility of glutathione in water. This structure of GSH provides an important advantage in the stability of the molecule and the defense of the molecule (4). In addition to being among the enzymatic systems responsible for the protection of intracellular redox balance, GSH plays critical roles in both the antioxidant defense system and many important metabolic events. Since the GSH level is high in many tumor types, the tissues become more resistant to chemotherapy (5). However, the GSH content of some types of tumor cells is associated with GSH-related

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enzymes such as γ-glutamyl cysteine ligase (GCL) and γ-glutamyl transpeptidase (GGT) enzymes. Because of this effect, GSH is seen as an effective agent in medical intervention for cancer progression and resistance to chemotherapy (6). In addition to being a multifunctional enzyme, the GST enzyme catalyzes the first step in the reaction sequence to form mercapturic acid, the final product in detoxification metabolism. Endogenous and exogenous hydrophobic electrophiles with GSH bind in the first reaction catalyzed by GST (7). In order for the GST enzyme to catalyze this reaction, GSH is required in the environment. Therefore, GSH is known as the cosubstrate of the GST enzyme. The GST enzyme is known as a partial substrate specific enzyme because its substrates other than GSH are widely distributed. In addition, they prevent the products formed by oxidation or foreign toxic substances from the combination with other macromolecules in the body and ensure that they are disposed of without damaging the cell components. Therefore, GSTs are one of the enzyme groups that act as a very important protection (8). The vast majority of cancer cell types contain much higher amounts of ROS and free radicals than healthy cells. In addition, it has been observed in many studies that the levels of antioxidant compounds and the amount of enzymes that destroy ROS increase in cancer tissues. The increase in the amount of enzymes that destroy ROS also creates a protective response in intracellular oxidative stres (9). There has been no significant improvement in preventing cancer, which is one of the most important causes of death in the world today (10). For centuries, people have treated their illnesses using natural products (11). Taking advantage of this, most anticancer substances have been developed using natural products. When developing new anticancer compounds, these anticancer agents are taken into consideration, either by replacing natural products or directly using natural products. Although many chemotherapy drugs of natural origin have been developed, new anticancer agents are still needed. Because this situation is necessary to find an effective method in cancer treatment (12). The biological activity of a compound depends on the molecular structure of that compound. Because of their widespread use in the design of drugs, the use of heterocyclic compounds is quite common in medicinal chemistry (13). Also, hetreocyclic compounds containing nitrogen in their structure are very important in drug development studies. The biological properties of pyrimidine compounds increase the importance of these compounds. From a clinical point of view; Pyrimidines are used in the treatment of many diseases such as anticancer, antiviral, cardiovascular, antidiabetic and anti-inflammatory drugs are used. Pyrimidine is the most common heterocyclic ring system found in nature. There is a pyrimidine ring in vitamin B1 (thiamine), riboflavin and folic acid. Uracil, thymine and cytosine are found in nucleic acids that form the essence of life, and their bases are pyrimidine derivatives. Also, there is a pyrimidine ring in folic acid. Drugs used in cancer chemotherapy are obtained from various derivative compounds derived from the pyrimidine ring (14). In the present study, the inhibition effects of some pyrimidine derivative compounds (4-amino-2-chloropyrimidine, 4-Amino-6-chloropyrimidine and 4-Amino-2,6-chloropyrimidine) (Figure 1) on the GST enzyme were investigated in vitro. For this purpose, firstly, GST enzyme was purified from erythrocyte using affinity chromatography technique. Later, with inhibition studies, inhibition type was determined together with IC_{50} and K_i value for pyrimidine derivative compounds.

Material and Methods

Chemicals: GST enzyme was purified from the erythrocytes obtained from the blood sample obtained from Blood Center. Except for the standard protein marker used for electrophoresis (Thermo Fisher Scientific), all chemicals used in all steps of the study were obtained from Sigma-Aldrich. (Taufkirchen, Germany).

Preparation of hemolysate from human erythrocytes: The study was started by centrifuging the blood sample at 1500xg for 15 minutes. Leukocyte, plasma and erythrocyte phases were formed in the tube after centrifugation. The erythrocyte phase was removed by discarding the plasma and leukocyte phases. Erythrocytes were washed 3 times with 0.9% NaCl and then rinsed in ice water for 30 minutes. The impurities were removed by centrifugation at 15.000xg for 30 minutes and the step of purification with the supernatant was started (15).

GST Activity assay: Measurement of GST enzyme activity was performed in a modified version of the method used by Habig et al. (16).

Enzyme purification: GSH-agarose was used as affinity gel for the purification of the GST

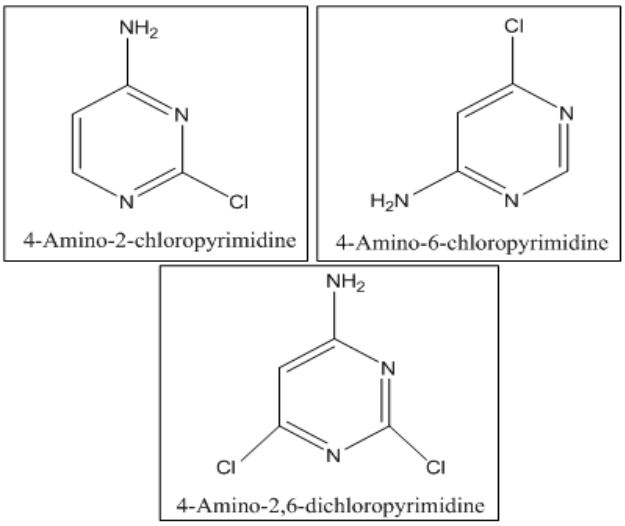

Figure 1. The molecular structure of pyrimidine derivatives used in inhibition studies

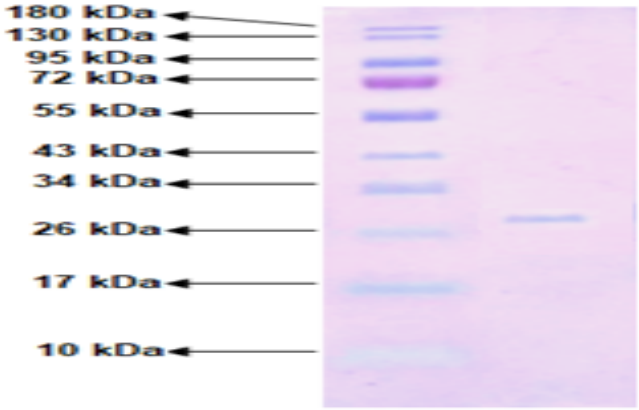

Figure 2. SDS-PAGE for GST enzyme

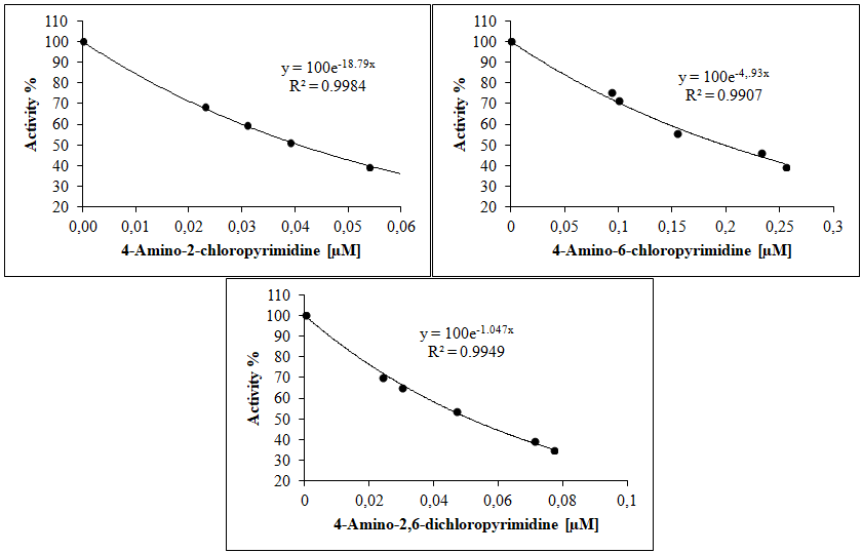

Figure 3. IC_{50} of pyrimidine derivatives on GST

Table 1: Summary of the GST purification procedure from human erythrocytes

Purification Steps	Total volume (mL)	Total protein (mg)	Total activity (EU)	Specific activity (EU/mg)	Purification fold	Yield (%)
Hemolysate	25	703.75	1.512	0.002	1	100
Affinity Chromatography	2	0.144	0.293	2.035	1017.36	19.38

Table 2: IC_{50}, K_i values and inhibition types of pyrimidine derivatives on GST

Pyrimidine Derivatives	IC_{50} (μM)	K_i (μM)	Inhibition Type
4-amino-2-chloropyrimidine	0.037	0.047±0,0015	Noncompetitive
4-amino-6-chloropyrimidine	0.139	0.272±0,1764	Noncompetitive
4-amino-2,6-dichloropyrimidine	0.662	0.076±0,0043	Noncompetitive

enzyme. The prepared hemolysate was loaded on the column. After hemolysate flowed through the column, the column was first equilibrated with equilibration buffer (10 mM phosphate buffer containing 150 mM NaCl, pH 7.4) and then washed using the same buffer. The GST bound to the column with elusion buffer (50 mM Tris-HCl buffer pH 9.0 and up to 10 mM GSH) was eluted and eluates in 1 mL volumes were collected. GST activity and protein amount were measured in the collected fractions. Fractions with activity were used in inhibition studies.

Protein quantity assay: The amount of protein was determined according to the Bradford method (17)

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE): The Laemmli method allowed the determination of the molecular mass of the enzyme as well as the purity control. The method was done as in previous studies (18)

In vitro pyrimidine derivatives studies: The inhibition effect of pyrimidine derivatives was investigated using five different inhibitor concentrations. Control cuvette had enzyme activity only, but no inhibitor added. The IC_{50} value was accepted as the inhibitor concentration causing 50% inhibition and this value for each inhibitor was calculated graphically. The K_I values of the compounds whose inhibition effects were examined by drawing the Lineweaver-Burk curve were calculated and the inhibition types were determined (18).

Results

This study was carried out to examine inhibition effects of some pyrimidine derivatives on GST enzyme activity. In this direction, firstly, The enzyme was successfully purified from human erythrocytes using affinity chromatography technique. Purification took place with 2.035 EU / mg protein specific activity and 19.38% fold (Table 1). As a result of SDS-PAGE, the enzyme was proved to be pure and the molecular mass of the enzyme was determined to be approximately 28 kDa. (Figure 2). IC_{50} values of 4-amino-2-chloropyrimidine, 4-amino - 2- chloropyrimidine and 4-amino-2-chloropyrimidine were found 0.037, 0.139 and 0.662 μM respectively (Figure 3). The study shows that K_i values of pyrimidine derivative compounds showing inhibition effect are 4-amino-2-chloropyrimidine (0.037 μM) > 4-amino-6-chloropyrimidine (0.140 μM) > 4-amino-2.6-dichloropyrimidine (0.662 μM). It was seen with the data obtained as a result of the study that these compounds inhibit the GST enzyme at micromolar level (Table 2). In the light of the results obtained, 4-amino-2-chloropyrimidine showed the best inhibition effect, while 4-amino-2.6-dichloropyrimidine showed the least inhibition. A non-competitive inhibition effect was observed for all substances whose effects were examined (Figure 4).

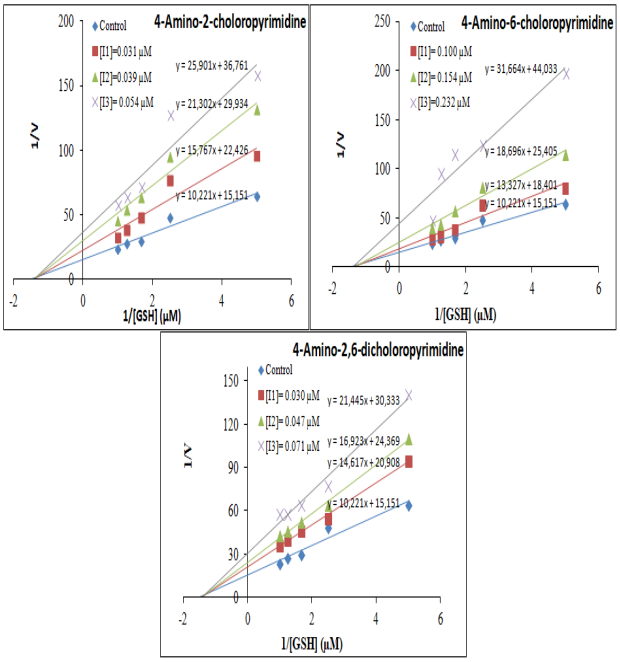

Figure 4. Lineweaver-Burk graphs for pyrimidine derivatives on GST

Discussion

Oxidative stress resulting from the production of excessive amounts of free radicals causes serious damage to the body. Irregular life or various disease states such as excessive and unbalanced food intake and irregular sleep result in chronic hyperglycemia and ketosis attacks. Due to the imbalanced conditions that occur between oxidants and antioxidants, DNA, proteins and lipids are damaged, causing cell death. Studies have revealed that oxidative stress causes the emergence of various diseases (19). The metabolic events of living organisms are related to the activity of enzymes that catalyze important reactions in their metabolism. Therefore, changes in enzyme activities are associated with the occurrence of many diseases. Due to this situation, it is desired to have useful agents in living organism for enzyme activity. Many drugs that are widely used clinically for treatment function by inhibiting enzymes. Therefore, while designing drugs, researchers are working on development by characterizing enzyme inhibitors that have the potential to be drugs that can inhibit specific enzymes. Therefore, the measurement of enzymatic activities in cells both in vivo and in vitro is a very important situation in drug discovery (20). Until today, many studies have been carried out by many researchers on GST enzyme activity. Taslimi et al. (21) conducted a study to examine the effect of benzene sulfonamide derivatives on the GST enzyme and found that the compounds inhibited the enzyme at micromolar level. In addition, Türkan et al. (22) found in their study that avermectin derivative group inhibited the GST enzyme at the mM level. In another study, Türkan et al. (23) investigated the effects of some cephalosporin antibiotics on the GST enzyme both in vivo and in vitro and found that the compounds inhibited the enzyme at millimolar level. Similarly, Gülçin et al. (24) investigating the inhibition effect of rosmarinic acid on the GST enzyme and it was found that rosmarinic acid inhibited the GST enzyme at nanomolar level. In another study, caffeic acid phenylethyl ester (CAPE) inhibited the GST enzyme at nanomolar level (25) In addition, in another study conducted by our team, the inhibition effect of chalcones on the GST enzyme was examined and the compounds examined in the study inhibited the enzyme at micromolar level (5). According to the results of the inhibition study, 4-amino-2-chloropyrimidine had a high inhibition effect on the GST enzyme. The amino and chlorine groups found in 4-amino-2-chloropyrimidine provided an effective inhibition. It was observed that although the chemical structures of 4-amino-2-chloropyrimidine and 4-amino-6-chloropyrimidine were exactly the same except for the binding position of the chlorine ion, the inhibition rates were quite different. Compared to IC_{50} values, 4-amino-2-chloropyrimidine showed 4 times more inhibition effect than 4-amino-6-chloropyrimidine. In this case, it was concluded that the binding position of the chlorine ion provides an effective inhibition for the GST enzyme. As a result, some pyrimidine derivatives are molecules that have been extensively studied by the scientific community for the treatment of various diseases and disorders. However, studies have not investigated the inhibition effects on the GST enzyme. For this purpose, the enzyme was purified in one step using affinity chromatography. Then, *in vitro* inhibition effects of some pyrimidine derivatives on this enzyme activity were investigated. It can be considered that pyrimidine derivatives may be effective on GST. The findings from this study make several contributions to the literature. These compounds, whose effect was investigated in the study and showed an inhibition effect on the GST enzyme, can shed light on further biological studies.

Aspect of the research ethic: For the research, on 31.03.2021, the ethics committee approval numbered 80576354-050-99/24 was obtained

from the Non-Invasive Clinical Research Ethics Committee of Kafkas University Faculty of Medicine. In addition, on 17.02.2020, research permission numbered E-85063915-929.99-4513 was obtained from Kafkas University Faculty of Medicine, Department of Basic Medical Sciences.

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Conflict of Interest: The author declared that there is no conflict of interest.

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