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Inhibition of human glutathione transferase P1-1 by novel benzazole derivatives

[İnsan glutatyon transferaz P1-1'in benzozol türevleri tarafından inhibisyonu]

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ABSTRACT

Objective: Glutathione transferases (GST) are multifunctional enzymes involved in detoxication, drug resistance, cell signaling and apoptosis. The inhibitory effects of novel benzazole derivatives were tested on human GST P1-1 to find new agents for overcoming drug resistance in cancer cells. **Methods:** GST P1-1 was heterogously expressed in E. coli strain XL-1 Blue and purified using S-hexylglutathione-Sepharose 6B affinity chromatography. The effect of 33 potential inhibitors on enzymatic activity was assayed spectrophotometrically with 1-chloro-2,4-dinitrobenzene (CDNB) as well as with the alternative substrate phenethyl isothiocyanate (PEITC). **Results:** Compound-18 (N-[2-(4-chloro-benzyl)-benzooxazol-5-yl]-4-nitro-benzenesulfonamide) was the most potent inhibitor found with an IC_{50} value of approximately 10 μM with respect to CDNB and a somewhat less strong inhibitor (45 % inhibition at 40 μM) with PEITC as substrate. Compound-18 showed mixed inhibition with GSH and uncompetitive inhibition with CDNB with the K_i values 6.3 ± 0.7 μM and 11.8 ± 3.4 μM, respectively.

Conclusion: Compound-18 is a potent inhibitor of GST P1-1. It may serve as a lead for further chemical modifications for increased potency. Additional studies will elucidate the effects of the inhibitor on cancer cells.

Key Words: GST P1-1, enzyme inhibition, benzazole derivatives, anti-cancer drugs

Conflict of Interest: The authors declare no conflicts of Interest of any kind.

ÖZET

Amaç: Glutatyon transferazlar detoksifikasyon, ilaç direnci, hücre sinyalizasyonu ve apoptozda görev alan çok fonksiyonlu enzimlerdir. Benzazol türevi bileşiklerin GST P1-1 üzerindeki inhibitör etkilerine bakılarak kanser hücrelerinde oluşan ilaç direncinin ortadan kaldırılmasını sağlayabilecek yeni inhibitörlerin bulunması hedeflenmektedir. **Yöntemler:** GST P1-1, E. coli suşu XL-1 Blue'da eksprese edidi ve S-hekzilglutatyon-Sefaroz 6B afinite kromatografisi kullanılarak saflaştırıldı. Toplam 33 tane bileşiğin potansiyel inhibisyon etkileri spektofotometre ile 1-klor-2,4-dinitrobenzen (CDNB) ve alternatif olarakta fenetil izotiyosiyanat (PEITC) substratı ile çalışıldı. **Bulgular:** Bileşik-18 (N-[2-(4-kloro-benzil)-benzoksazol-5-il]-4-nitro-benzensülfonamit), GST P1-1 üzerinde yaklaşık 10 μM IC_{50} değeri ile CDNB'ye göre kuvvetli bir inhitör, PEITC göre de daha az kuvvetli bir inhibitör (40 μM'da % 45 inhibisyon) bileşiktir. Ayrıyeten GSH substratına göre karışık tip inhibisyon (K_i^{GSH} 6.3 ± 0.7 μM), CDNB'ye göre de unkompetitif inhibisyon (K_i^{CDNB} 11.8 ± 3.4 μM) göstermektedir. **Sonuç:** Bileşik-18 GST P1-1 için kuvvetli bir inhibitördür. Bu molekül inhibisyonun potansiyel etkisini artırmada kimyasal modifikasyonlar için öncül bileşik olabilir. İnhibitörün kanser hücrelerinde etkilerini açığa çıkartabilmek için ileri çalışmalara ihtiyaç duyulmaktadır.

Anahtar Kelimeler: GST P1-1, enzim inhibisyonu, benzazol türevleri, anti-kanser ilaçlar.

Çıkar Çatışması: Yazarlar arasında çıkar çatışması bulunmamaktadır.

Introduction

Glutathione transferases (GSTs; EC 2.5.1.18) are multifunctional enzymes that are involved mainly in detoxication of endogenously produced and xenobiotic compounds in living organisms. These enzymes catalyze the conjugation of the reduced form of glutathione (GSH) to electrophilic centers of hydrophobic compounds [1]. GSTs are found in almost all organisms from mammals to plants and even in some prokaryotes. In mammalian cells seven classes of cytosolic GSTs (termed alpha, mu, pi, sigma, zeta, omega and theta) have been identified in dimeric forms [2]. Each subunit contains two active sites; one for specific binding of GSH and the other for relatively nonselective binding of hydrophobic substrates [3].

GSTs comprise a large family of isozymes with alternative functions. Apart from detoxication they also catalyze isomerase reactions in the metabolism of steroid hormone biosynthesis and amino acid degradation reactions [4, 5]. In addition, GSTs also have noncatalytic roles. They may function in the intracellular transport of non-substrate compounds like bilirubin, heme and steroids [6, 7] and have regulatory roles in cell signaling and apoptosis through the inhibition of c-Jun-N-terminal kinase [8].

GST P1-1, the most prevalent isoform in mammalian organisms, is involved in the development of resistance to anti-cancer cells towards drugs, herbicides and pesticides [9-11]. It is suggested that this resistance is related to high expression of GST P1-1 in cancers such as breast, lung, colon pancreas and cervix thereby contributing to resistance to chemotherapy [12]. In order to overcome this resistance specific GST P1-1 inhibitors are in demand.

A variety of compounds can be identified in the literature as inhibitors of GST P1-1. Ethacrynic acid, TLK-286, 6-(7-nitro-1,2,3-benzoxadiazol-4-ylthio) hexanol (NBDHEX), Cibacron blue and bromosulfophthalein are known amongst the most potent ones [*3, 13*]. Of these, TLK-286 is a promising compound tested in Phase III clinical trials for ovarian cancers [14] and NBDHEX is a compound that may block interactions of GST P1-1 with c-Jun N-terminal kinase (JNK). However, additional inhibitors are in demand, since the toxicity of the known compounds limits their use in clinical and in vitro studies [13].

In the present study the inhibitory effects of a series of novel compounds containing benzothiazole and benzoxazole groups were studied with human GST P1-1. According to our results compound-18 (N-[2-(4-chloro-benzyl)-benzooxazol-5-yl]-4-nitro-benzenesulfonamide) was the most potent inhibitor. Further analysis and kinetic details are discussed.

Materials and Methods

Synthesis of compounds

The benzazole compounds containing benzothiazole and benzoxazole rings with small differences in the side chains were obtained from Ankara University Faculty of Pharmacy Department of Pharmaceutical Chemistry. The synthesis of these totally 33 benzazole compounds were published before [15-22].

Expression and purification of human GST P1-1

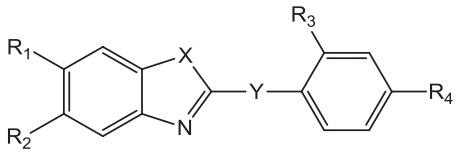
Recombinant hGST P1-1 was expressed in *Escherichia coli* strain XL-1 Blue at 37°C and purified by using S-hexylglutathione-Sepharose 6B [*23*]. E.coli XL-1 cells containing pKXHP1 plasmid were grown overnight in 50 ml 2YT media (16 g tryptone, 10 g yeast extract, 5 g NaCl and 100 mg/liter ampicillin) and then transferred to 500 ml 2YT media and incubated at 37 °C in the shake incubator. Incubation was kept until the absorbance of the culture at 600 nm was 0.2-0.4. Then 0.2 mM isopropyl-β-D-thiogalactopyranoside was added to induce the expression of GST P1-1. The cells were incubated for 16 hr and then centrifuged at 7000 rpm for 7 minutes. The pellets were kept at -80 °C for 30 minutes. The pellets were resuspended in lysis buffer (10 mM Tris HCl, 1 mM EDTA, 0.2 mM dithiothreitol (DTT) pH 7.0 and protease inhibitor cocktail, 0.2 mg/ml lysozyme) and mixed gently on ice for 30 min and then disrupted by sonication 5 times for 20 seconds. Phenylmethanesulfonylfluoride (170 μM) was added and the supernatant fraction was obtained by centrifugation at 15000 rpm, 5 °C for 1 hr.

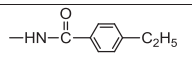
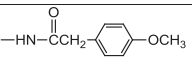
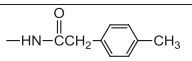
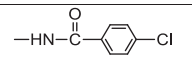
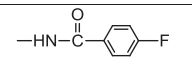
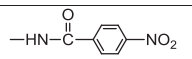
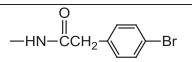
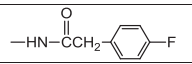
Epoxy-activated S-hexylglutathione-Sepharose 6B affinity matrix was used for purification of GST P1-1. The supernatant fraction was applied to the matrix equilibrated with binding buffer and stirred gently for 40 minutes on ice. The matrix containing bound GST P1-1 was washed with Buffer A (10 mM Tris HCl pH 7.8 1 mM EDTA, 0.2 M NaCl, 0.2 mM DTT) to eliminate non-bound proteins. The matrix was packed on top of a Sephadex G-25 column equilibrated with Buffer A in the cold room. The enzyme was then eluted with Buffer B (10 mM Tris HCl pH 7.8, 1 mM EDTA, 0.2 M NaCl, 0.2 mM DTT, 5 mM S-hexylglutathione). The fractions containing the GST activity were concentrated on ice and then dialyzed with Buffer A without NaCl. The purity of the enzyme was determined by SDS-PAGE applying both optimal and excessive protein amounts for analysis to visualize possible impurities.

GST assay and inhibition studies

GST activity was measured on a Shimadzu UV-2501 PC spectrophotometer by measuring the initial rate of absorbance change at 340 nm with CDNB [24]. Standard enzymatic assay conditions consisted of 0.1 M phosphate buffer, pH 6.5 containing 1 mM EDTA, 1 mM GSH, and 1 mM CDNB at 30 °C. The reaction system contained 5 % ethanol (from the CDNB stock solution), but the solvent had a negligible inhibitory effect on the enzyme activity. The enzymatic reaction was obtained by subtracting the nonenzymatic rate from the rate measured in the presence of enzyme.

Table 1. The structure of tested compounds and the inhibitory effects.



No	X	Y	R_1	R_2	R_3	R_4	GST P1-1 inhibition	Reference
1	S	-	H	H	H		none	[15]
2	S	-	H	H	H		none	[15]
3	S	-	H	H	H		none	[15]
4	S	-	H	H	H		none	[15]
5	S	-	H	H	H		none	[15]
6	S	-	H	H	H		none	[15]
7	S	-	H	H	H		20 μM → 60 % inh.	[15]
8	S	-	H	H	H		20 μM → 58 % inh.	[15]
9	S	-CH_2O-	H	H	H	H	None	[16]

The most potent compounds identified with CDNB were also tested spectrophotometrically with PEITC as substrate in a standard assay [25]. The molar absorption coefficient used for CDNB was $\Delta\varepsilon_{340}$ = 9.6 mM^{-1} cm^{-1} and for PEITC $\Delta\varepsilon_{274}$ = 8.89 mM^{-1} cm^{-1}. The IC_{50} value was determined as the concentration of the inhibitor that gives 50% inhibition of the enzymatic activity. For determining the inhibition type of compounds varied concentrations of GSH and CDNB were used. The compounds tested as inhibitors were prepared freshly in 2 mM stock solutions in 96% ethanol because of their sensitivity to light.

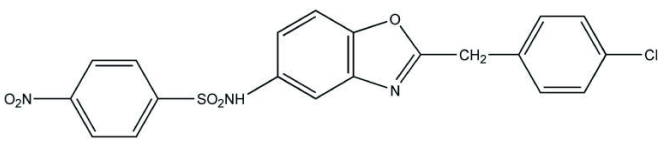


Figure 1. The chemical structure of compound-18 N-[2-(4-chloro-benzyl)-benzooxazol-5-yl]-4-nitro-benzenesulfonamide.

Data Analysis

All measurements were made in triplicate and each point on the graphs was given with standard deviation of the mean value. IC_{50} and K_i values of the compounds were determined by regression analysis using Graphpad Prism 4.0 software.

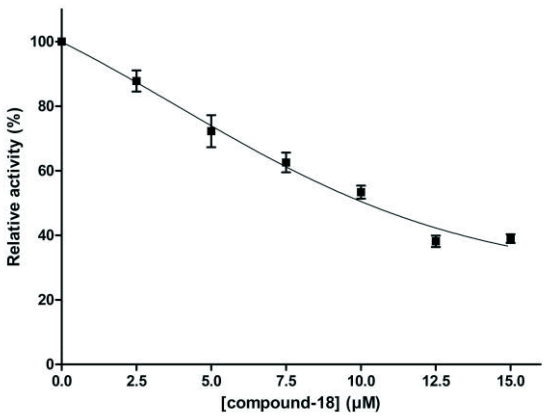


Figure 2. The inhibition profile of compound-18 on GST P1-1. The enzyme activity was determined in the presence 1 mM GSH, 1 mM CDNB at 30 °C under standard assay conditions.

Results

Expression and purification of hGST P1-1

GST P1-1 was expressed in E. coli at 37 °C and purified by using S-hexylglutathione-Sepharose 6B. A total yield of 40 mg of protein per liter culture was obtained. The specific activity of the purified protein was 100 μmol min-1 mg-1 with CDNB as substrate. The final stock

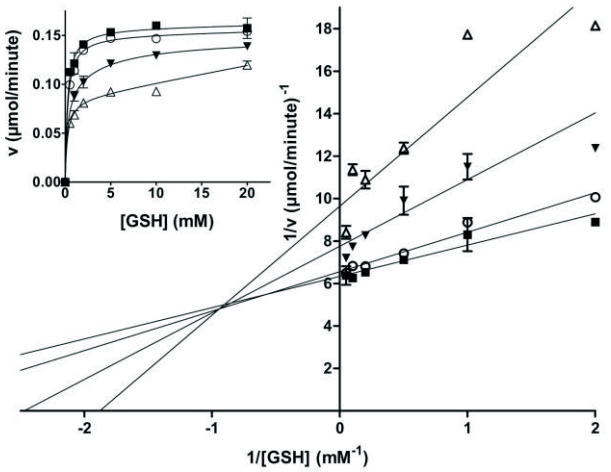


Figure 3. Inhibition profile of compound-18 on GST P1-1 versus GSH. In the assay GST P1-1 activity was measured with varying concentrations of GSH and different inhibitor concentrations: (■) 0 µM, (○) 2.5 µM, (▼) 5 µM and (Δ) 10 µM. The electrophilic substrate CDNB was used at 1 mM concentration, which is near its solubility limit and close to half-saturation of the enzyme (cf. Figure 4).

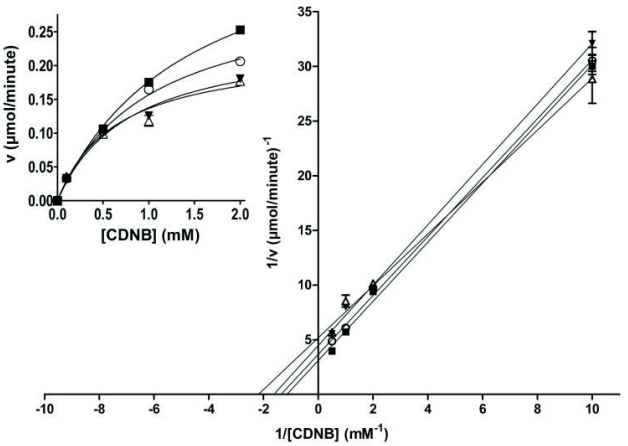


Figure 4. Inhibition profile of compound-18 on GST P1-1 versus CDNB. In the assay GST P1-1 activity was measured with varying concentrations of CDNB and different inhibitor concentrations: (■) 0 µM, (○) 5 µM, (▼) 10 µM and (Δ) 15 µM. The first substrate GSH was used at 5 mM concentration, which is near half-saturation of the enzyme (cf. Figure 3).

solution of purified GST P1-1 had a concentration of 5.47 mg/ml and was stored frozen at -80 °C.

In vitro Inhibition Analysis of Compounds

The structures, names, and the inhibition results of the benzoxazole- and benzothiazole-group containing compounds are given in Table 1. The GST P1-1 activity was monitored at 30 °C for one minute in the standard CDNB assay. The compounds yielding IC50 values below 10 µM were considered to be potent inhibitors and subjected to further kinetic analysis to obtain Vmax, Ki and inhibition type.

Seven of the 33 novel compounds tested as inhibitors gave significant effects under the standard assay conditions (Table 1). According to our results compound-18 was the most potent inhibitor among the compounds studied (Figure 1). It inhibited GST P1-1 with an approximately 10 µM IC50 value in the assay system with CDNB as substrate (Figure 2). The inhibition analysis was also carried out with PEITC as an alternative substrate. Compound-18 showed 45 % inhibition at 40 µM with the substrate PEITC. (In the assay compound-18 was not used above 40 µM concentration because of limited solubility in the assay).

The inhibition type of compound-18 with respect to varying concentrations of GSH and CDNB, respectively, was also determined. Figure 3 shows substrate-saturation and Lineweaver-Burk plots with GSH as the varied substrate. As a first crude approximation compound-18 shows mixed inhibition with a Ki value of 6.3 ± 0.7 µM. However, closer examination suggests that compound-18 induces a deviation from linearity in the Lineweaver-Burk plot; the graphs corresponding to different inhibitor concentrations may in fact converge on the 1/v-axis indicating competitive inhibition with GSH. By contrast, the graphs in the Lineweaver-Burk plot with respect to CDNB indicate uncompetitive inhibition with a Ki value of 11.8 ± 3.4 µM (Figure 4). The results and the kinetic parameters are summarized in Table 2.

Discussion

Inhibitors for GSTs have been investigated for more than forty years for different purposes. In the beginning they were used to understand the catalytic mechanism

Table 2. IC_{50}, K_i and inhibition type of compound-18 in the CDNB assay of GST P1-1.

			Inhibition type	
Compound	K_i^{GSH} (μM)	K_i^{CDNB} (μM)	GSH	CDNB
Compound-18	6.3 ± 0.7	11.8 ± 3.4	mixed	uncompetitive

and the active site topography of the enzymes, and even to distinguish between some of the isoenzymes in the GST family [26, 27]. However, today more advanced techniques like crystallography, mass spectrometry and gene sequencing are more accurate to reveal these functions.

In recent years, studies of GSTs have revealed new roles for some of the members of this family. It has been demonstrated that GSTs of classes alpha, mu, and pi are involved in cell proliferation, differentiation and control of cell death via interactions with special signaling proteins [28-30]. In particular, GST P1-1 is overexpressed in some cancer cells and it has been suggested to cause drug resistance [31]. Therefore, the use of inhibitors to suppress the GST P1-1 activity in cancer cells is a promising method to overcome such drug resistance.

In the present study novel benzazole compounds containing benzoxazole and benzothiazole rings were examined for their effects on hGST P1-1 activity. According to our results some of the benzazoles with the benzoxazole ring exhibit inhibitory effects on hGST P1-1 (Table 1). Among these compounds the most potent inhibitor is compound-18 with an approximately 10 μM IC_{50} value according to CDNB substrate. This IC_{50} value is quite low and the compound was selected for further kinetic studies. For comparison earlier studied inhibitors give IC_{50} values of 5 μM for hematin, 6 μM for tributyltin bromide, and 20 μM for S-hexylglutathione [26].

In inhibition studies, it is useful to investigate the effects of the compounds with more than one substrate. For example, progesterone is not a inhibitor of GST P1-1 assayed with CDNB, but when ethacrynic acid is used as the substrate progesterone is a potent inhibitor (1.4 μM of IC_{50}) [32]. For this reason, the inhibitory effect was also studied with PEITC as substrate to evaluate the potency of the compound. Compound-18 shows an apparently somewhat lower potency versus PEITC with 45 % inhibition at 40 μM inhibitor concentration. On the other hand, the differences are not major considering that the PEITC concentration used (400 μM) corresponds to approximately 80% substrate saturation (based on a K_m values of 120 μM; [25]) as compared to 50% saturation with CDNB.

Further kinetic studies were done to determine how the inhibitor binds to GST P1-1 enzyme. Compound-18 is a mixed inhibitor with GSH and an uncompetitive inhibitor with CDNB characterized by K_i values 6.3 ± 0.7 and 11.8 ± 3.4 μM, respectively. This information indicates that compound-18 interacts not only with the GSH-binding site but also to other regions of enzyme.

Chemotherapeutic drugs can be used either alone or in combination with other compounds to counteract drug resistance in cancers [33, 34]. For both regimens, compound-18 is a good candidate to test on cancer cells. In initial studies, breast cancer cells with high expressions of GST P1-1 can be targeted in vitro with compound-18 alone to examined for effects on cell survival and apoptosis. In combination with alkylating drugs, known to be metabolized by GST P1-1, compound-18 can be tested for suppression of drug resistance.

In conclusion, we identified compound-18 as a potent inhibitor for GST P1-1. This is the first benzoxazole derivative published in literature as an inhibitor for GSTs. Further kinetic studies including inhibition effects on other GST isoenzymes are necessary to establish selectivity for GST P1-1. Structural studies by crystallography or other methods are needed to understand the particular interactions providing binding specificity of the compound. Using this information more effective compounds can be designed for in vitro and in vivo studies.

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