Turk J. Pharm. Sci. 9(2), 151-160, 2012

Original article

INDOLE DERIVATIVES AS SRC FAMILY KINASE AND GLUTATHIONE S-TRANSFERASE INHIBITORS: EVALUATION OF THEIR SELECTIVITY AND DRUG RESISTANCE PROPERTIES

Belgin S. İŞGÖR[1], Zühal KILIÇ KURT[2], Yasemin G. İŞGÖR[1], Süreyya ÖLGEN[2,*]

[1]Atılım University, Faculty of Enginerring, Chemistry Group, 06836 İncek, Ankara, TURKEY
[2]University of Ankara, Faculty of Pharmacy, Department of Pharmaceutical Chemistry, 06100 Tandoğan-Ankara, TURKEY

Abstract

The increased activity levels of Glutathione S-transferases (GST) have been correlated with human cancers and the anticancer drug resistance. Similarly, Src family kinases (SFKs), has been reported in many cancers including breast, colon, lung, and skin with relatively high catalytic activity. Therefore, the inhibition of both GSTs and Src may enhance the therapeutic efficacy of chemotherapeutics by increasing the selectivity and resistance of compounds. The recent efforts of our laboratory to design and synthesize novel c-Src inhibitors were accomplished with four indole-3-amine derivatives substituted at N1 and C5 (8c, 8f, 8g, and 8h), with IC_{50}s values of 4.69, 74.79, 75.06, and 84.23 μM, respectively. In this present work, the inhibitory activities against SFKs (Lyn, Hck, Fyn), GSTs and selectivity studies of these compounds were performed. Among the compounds, 8c and 8g are found the best GST inhibitors with IC_{50}s values of 120.1, and 67.33 μM, respectively, and are reported as the Src inhibitors with dual action. The compounds 8f and 8h are also showed reasonable inhibitory levels of GSTs with IC_{50}s of 161.1, and 272.2 μM, However inhibition profiles of compounds are not found suitable for further developments.

Key words: *Glutathione S-transferase, Src kinases, Indole derivatives, Dual inhibitors.*

Glutatyon S-transferaz ve Src Ailesi Kinaz İnhibitörü İndol Türevleri: Seletivite ve İlaç Rezistans Özelliklerinin Değerlendirilmesi

Artan glutation s-transferaz (GST) aktivitesi insanlarda görülen kanserler ve antikanser ilaç direnci ile ilişkilidir. Benzer şeklilde, Src ailesi kinazların (SFK) meme, kolon, akciğer ve deri gibi pek çok kanser türünde yüksek katalitik aktivitesi olduğu rapor edilmiştir. Bu nedenle, GST ve Src'ın her ikisinin inhibisyonu, kemoterapötiklerin seçici ve dayanıklı olmasını sağlayarak terapötik etkinliğini artırabilir. Son yıllarda laboratuvarımızda gerçekleştirilen c-Src inhibitörü bileşiklerin tasarımı ve sentezi çabaları ile IC_{50} değeri sırayla 4.69, 74.79, 75.06 ve 84.23 μM olan N1 ve C5 sübstitüe indol-3-amin türevi dört bileşik (8c, 8f, 8g ve 8h) başarı ile elde edilmiştir. Bu çalışmada, bu bileşiklerin SFK'lar (Lyn, Hck, Fyn) ve GST enzimlerine karşı inhibitör aktiviteleri tayin edilmeye ve Src ailesi kinazlara olan seçicilikleri saptanmaya çalışılmıştır. Bileşiklerden 8c and 8g GST enzimi için sırayla IC_{50} değerleri 120.1 ve 67.33 μM olan en etkin inhibitörler ve ayrıca Src inhibitör özelliklerinin olması de ikili inhibitörler olarak rapor edilmişlerdir. Bileşiklerden 8f ve 8h sırayla IC_{50} değerleri 161.1 ve 272.2 μM ile önemli derecede GST inhibisyonu gösterdiler. Ancak bileşiklerin bu inhibisyon profilleri ile ileri çalışmalar için uygun olmadıkları bulunmuştur.

Anahtar kelimeler: *Glutation-S-transferaz, Src kinazlar, indol türevleri, ikili inhibitörler.*

*** Correspondence:** E-mail: olgen@ankara.edu.tr, Tel: +90 (312) 203 30 73 Fax: +90 (312) 213 10 81.

INTRODUCTION

Src family kinases (SFK), as members of nonreceptor tyrosine kinases (1), consist of highly homologous proteins, namely, Blk, Brk, Fgr, Frc, Fyn, Hck, Lck, Lyn, c-Src, Srm, and Yes. Among these proteins c-Src (other namely Src) is the first discovered and studied non-receptor protein tyrosine kinases (PTKs), and also the protypical member of SFKs (2-4). As a result of mutational studies and structural modeling based on crystallography data, the structure of Src has been well characterized (Figure 1). It contains Src homology domain 4 (SH4), a unique domain, SH3 domain, SH2 domain, a catalytic domain (SH1), and a C-terminal regulatory region (5). Src kinase domain, which is the harbor of the kinase active site, plays an important part in Src regulation along with the SH2 and SH3 domains. Src has two major phosphorylation sites, tyrosine 416 and tyrosine 527. The mechanism involved in the activation of Src is achieved by auto-phosphorylation of tyrosine 416, which is then displaced from the binding pocket (6). Src inhibitors can be classified into two major groups based on the mechanisms of action (7): 1) Inhibitors of tyrosine kinase activity, 2) Inhibitors of protein-protein (SH2-, SH3-, substrate binding domain-mediated) interaction. Most Src inhibitors discovered belong to the first group, which are more promising in terms of selectivity and potency. Src plays a role in several signalling pathways that are involved in cell proliferation and survival. Additionally, the ability of Src to promote tumor cell invasion can lead to the development of tumor metastasis (8). Collectively, Src plays a major role in regulating important mechanisms of specific receptor pathways where their activation can influence the biological activities of the tumor cell (9).

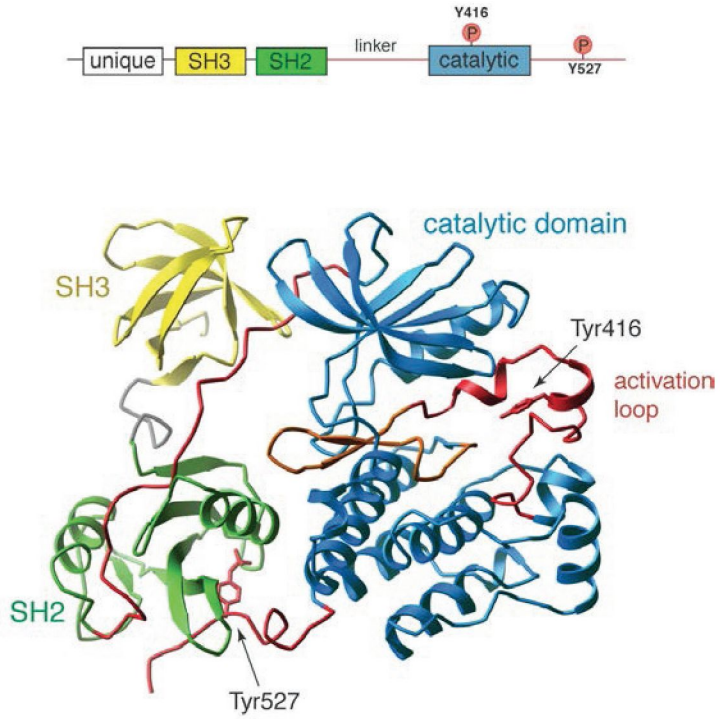


Figure 1. Ribbon diagram illustrating the structure of human Src. Key phosphorylation sites are included.

Being involved in various mechanisms in cellular functioning, deregulation of SFK signaling found explicit in the development and progress of multiple human cancer types in tissues including breast, gastrointestinal system, lung, ovary, pancreas, prostate, and skin (10, 11). Therefore SFK members became universal molecular targets and the efforts to discover

novel small molecules targeting SFKs received significant attention for developing novel therapeutics (12). With an emphasis on combination therapies with standard chemotherapeutic agents, the selectivity of SFK inhibitors may occur by increasing the sensitivity of tumors to established chemotherapeutic agents and by preventing tumors to metastases (13). The drug resistance of tissues is also found associated with the Glutathione S-transferases (GSTs), a family of phase II detoxification system enzymes (14). Many endogenous or xenobiotic lipophilic substances are eliminated from the cells by the sequence of oxidation, conjugation to an anionic group (glutathione, glucuronate or sulfate) and transport across the plasma membrane into the extracellular space GSTs are capable of recognize diverse chemical structures, catalyze their conjugation to glutathione (GSH), and hence reduce cytotoxic reactivity of those chemicals (Figure 2). These enzymes are generally considered to be detoxification of both endogenous and xenobiotic compounds; however, GSTs can also lead to the formation of more reactive intermediates and may cause the deregulated signaling which may predispose malignant transformation (15). Besides catalyzing the inactivation of various electrophile-producing anticancer agents via conjugation to the glutathione, GSTs are promising negative regulator for oxidative stress and drug-induced cell apoptosis through the interaction with specific signaling kinases (16). On the other hand, the multidrug resistance-associated proteins (MRPs) known to have potential role in clinical multidrug resistance, transfer the glutathione conjugates out of cells to prevent the accumulation of anionic conjugates and therefore, have critical and an essential role in defense against oxidative stress (17). In this context, for effective therapeutic efficacy it seems critical to have dual targeting capacity of inhibitors aimed at a specific enzyme responsible in disease progress and those responsible in developing drug resistance in that target tissue (18).

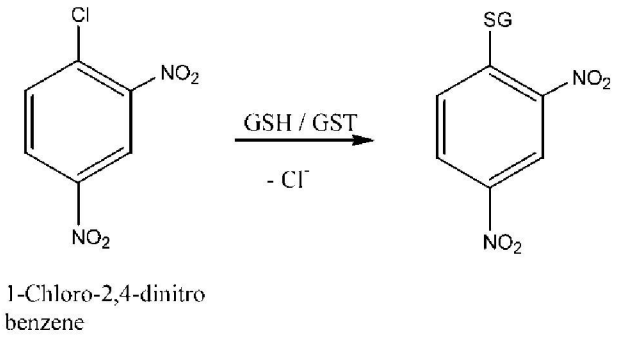


Figure 2. GST Conjugation Assay.

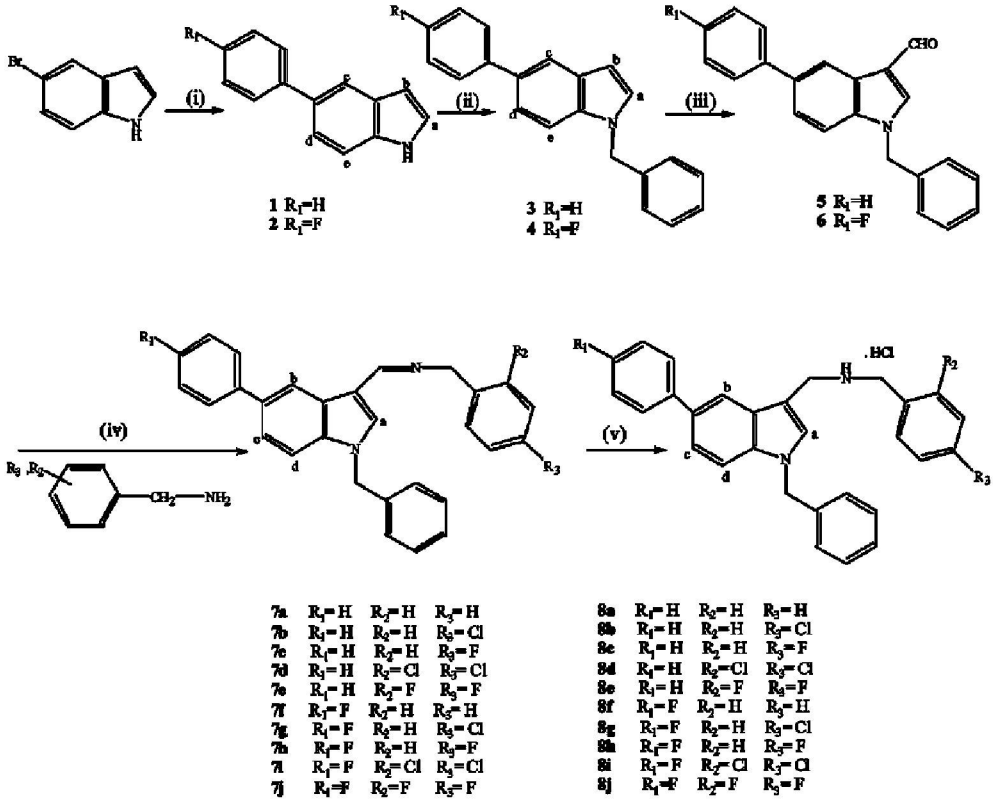
Here, we aim to evaluate previously found c-Src active indole-3-amine derivatives substituted at N1 and C5 with respect to their inhibitory activity and selectivity against Src family kinases, namely Fyn, Hck and Lyn, and GSTs to identify promising inhibitors that may have dual function by inhibiting the specific molecular target (Src kinases) and reducing the drug resistance capacity simultaneously. In this present work, their inhibitory efficacy on GSTs is shown by virtue of kinetic studies with bovine liver cytosol, and on SFKs by fluorescent labeled substrate with recombinant SFKs.

EXPERIMENTAL

Chemistry

The synthesis of tested compounds indole-3-amine derivatives substituted at N1 and C5 were reported in our previous publication (Scheme 1) (19). Briefly, palladium catalyzed cross-coupling reaction (Suzuki coupling reaction) between bromoindole and arylboronic acids provided starting compounds 5-phenyl indole (1) and 5-(p-fluorophenyl)indole (2), which were benzylated at indole nitrogen (compounds 3 and 4) following standard procedures using NaH 95% in dry DMF. After preparing carbaldehyde derivatives (5 and 6) with Vilsmaier formylation, they were then subjected to a condensation with substituted benzylamines in CH_2Cl_2 resulting in the Schiff' bases as Z / E (syn / anti) isomer mixtures (7a-j). The reaction of imine compounds with $NaBH_4$ afforded their oily amine congeners, which were converted into a solid form as HCl salts (8a-j).

Reagents and conditions: (i) $Pd(PPh_3)_4$, Na_2CO_3, arylboronic acids, anhydrous toluene; (ii) NaH, BnBr, DMF, 0°C to room temperature; (iii) DMF, $POCl_3$, –15°C to room temperature; (iv) substituted benzylamines, $MgSO_4$, CH_2Cl_2, reflux; (v) Na_2BH_4, 50°C.



Scheme 1. Synthesis of 1,3,5-trisubstituted indole derivatives (19)

Assay for glutathione S-transferase

The method of Habig to measure the kinetic change in substrate GSH utilization by GST were slightly modified and adopted for micro scale applications (20-22). Briefly, the total GST

activity was measured in a 100 mM potassium phosphate buffer at pH 6.5 with 2.4 mM 1-chloro-2,5-dinitro benzene (CDNB) and 3.2 mM GSH. In the assay, the compounds to be tested or DMSO (vehicle control), CDNB and reduced GSH were transferred to microplate in reaction buffer (100 mM potassium phosphate buffer at pH 6.5) and incubated at room temperature (22-25°C) for 5 minutes. Upon addition of enzyme source (bovine liver cytosol) microplate transferred to spectrophotometer and GSH-CDNB conjugate formation (Figure 2) was monitored as an increase in the absorbance at 340 nm for 240 seconds. Initial rates of enzymatic reactions were determined as nanomoles of the conjugation product of GSH and reported as nmole/minute/mg protein, where the total protein content was determined. In the analysis of compounds the kinetic measurement performed but the calculations were made with respect to the vehicle control. All measurements were performed at 340 nm, in 96 well microplate, using Spectramax M2e.

Assay for Src family tyrosine kinase activities

The activity measurement was performed using ProFluor Src-Family Kinase Assay protocol (Promega) with some modifications of the manufacturer's protocol (23). The pure enzyme c-Src (Invitrogen) was used at certain activities that yield 20% of the maximum fluorescent signal and the compounds to be tested were used in final concentrations in appropriate buffer with 0.5% DMSO. Test compounds were added to 96-well plate, except for 16 control wells that receive only the vehicle. Kinase solution (contains Src-Family Kinase R110 substrate) was added to all wells. To initiate the kinase reaction, ATP solution was added to each wells, except for 8 controls that receive control buffer. After incubating the plate at room temperature (22-25°C) for 60 min, protease solution was added to each well and incubated again for 60 min at room temperature. Stabilizer solution was added, mixed and the fluorescence was readed at excitaition wavelength of 485 nm and an emission wavelength of 530 nm. The decrease in fluorescence of each well inversely relates to kinase activity of the enzyme within the wells and comparison performed with respect to control wells. The assay is optimized for SFKs, namely Fyn, Hck and Lyn, as well as compound background fluorescence interferences. The IC_{50} values were determined by nonlinear regression analysis, the four parameter logistic equation (Sigmoidal dose–response, GraphPad Prism version 4.0 for Windows, GraphPad Software).

RESULTS AND DISCUSSION

In this study, the inhibitory activities against Src family kinases (Lyn, Hck, Fyn) and GSTs of c-Src active amine derivatives substituted at N1 and C5 compound were determined. The kinase reactions were performed with enzyme activities yielding 20% of the maximum flurescence signal for R110 assay, and 80% of phospho-peptide signal for ELISA at 40-100 nM ATP. Briefly, the assay begins with a standard kinase reaction performed in the provided reaction buffer (contains Src-family kinase R110 substrate). As shown in Figure 3, the substrate is nonfluorescent. Following the kinase reaction, addition of a termination buffer stops the kinase reaction and removes amino acids specifically from the non phosphorylated substrate, resulting in the production of highly fluorescent rodamine 110. Thus, the measured fluorescence intensity is inversely correlated with kinase activity and can be used for the determination of kinase activity and potency of inhibitors. The glutathione transferase reactions were performed with 925.6 unit/ml at 2.4 mM CDNB and 3.2 mM GSH concentrations.

Compound 8c and 8f seem to inhibit only Fyn and GST, as well as c-Src (Table 1, Figures 4 and 7). Compound 8g and 8h inhibit Fyn, Hck, Lyn (Figures 4-6). Compound 8g is the best inhibition of Lyn and GST among four tested compounds. All those compounds studied here were shown previously (19) as c-Src inhibitors within the range of 4-84 μM IC_{50}s values in Table 1.

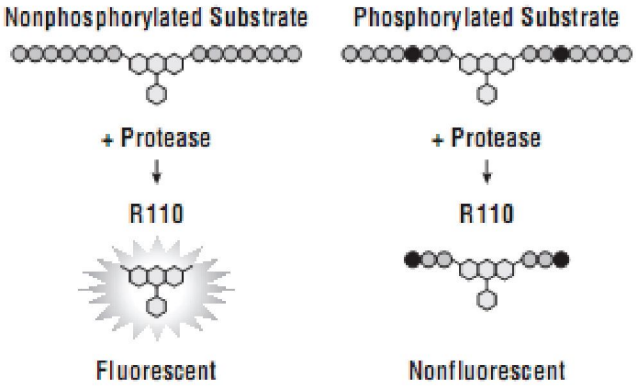


Figure 3. Rhodamine 110 based kinase/phosphatase assay.

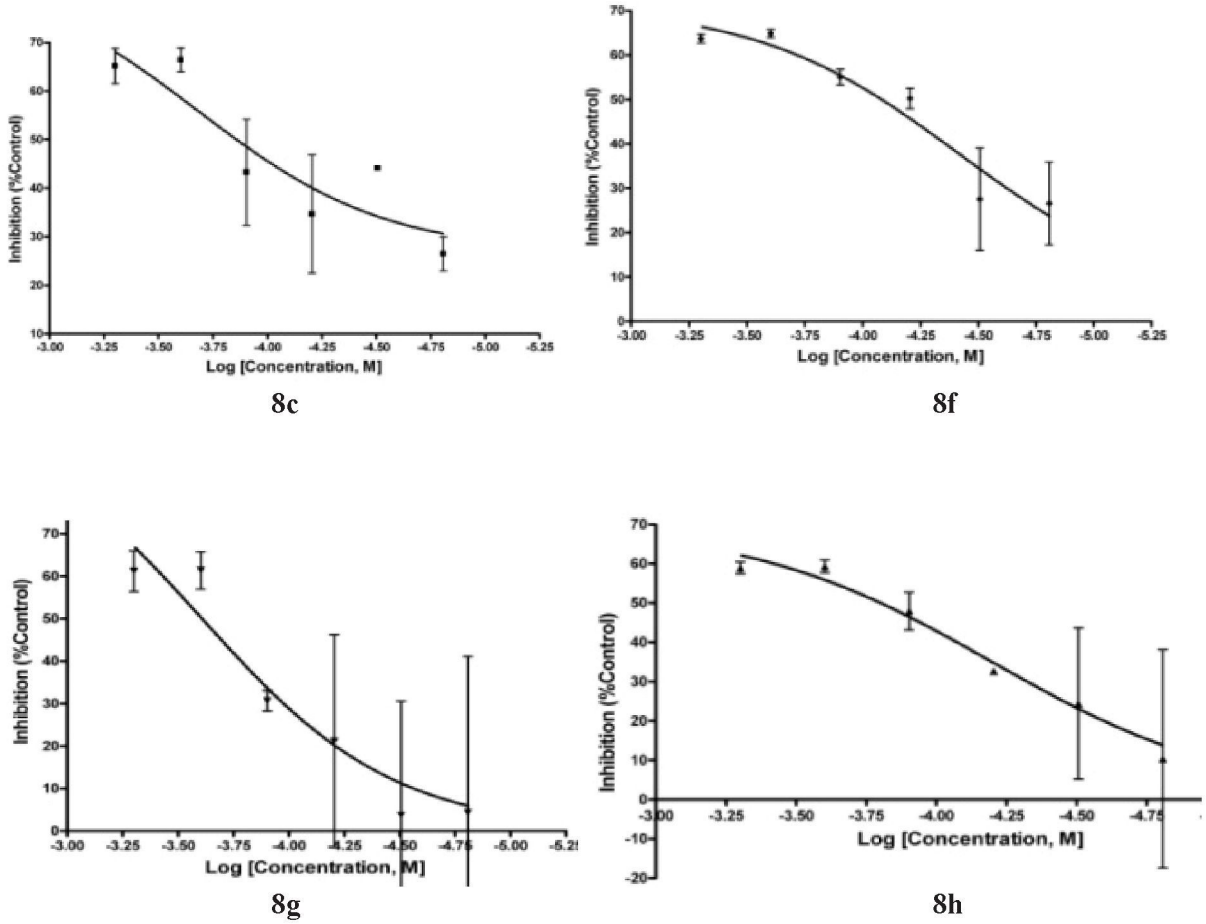


Figure 4. Fyn kinase inhibition of compounds 8c, 8f, 8g, and 8h. Data shown here is the prescreening result of compounds at two different concentrations, each in duplicate.

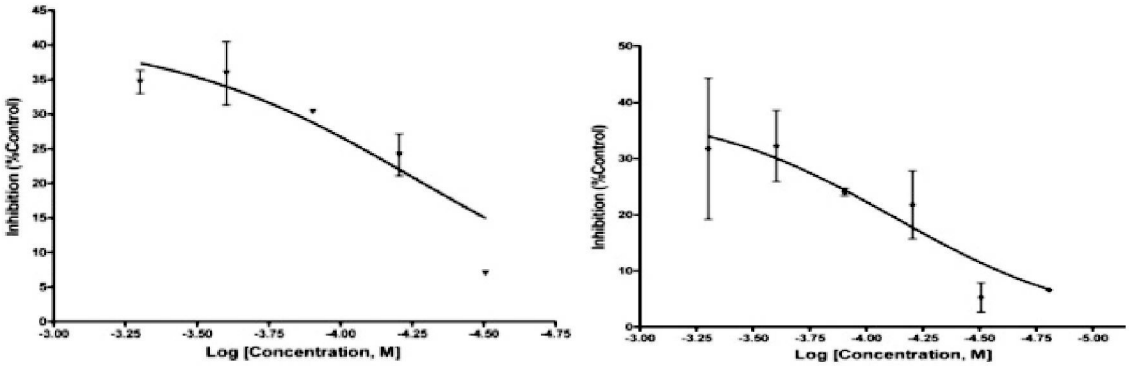


Figure 5. Hck kinase inhibition of compounds 8g and 8h. Data shown here is the prescreening result of compounds at two different concentrations, each in duplicate.

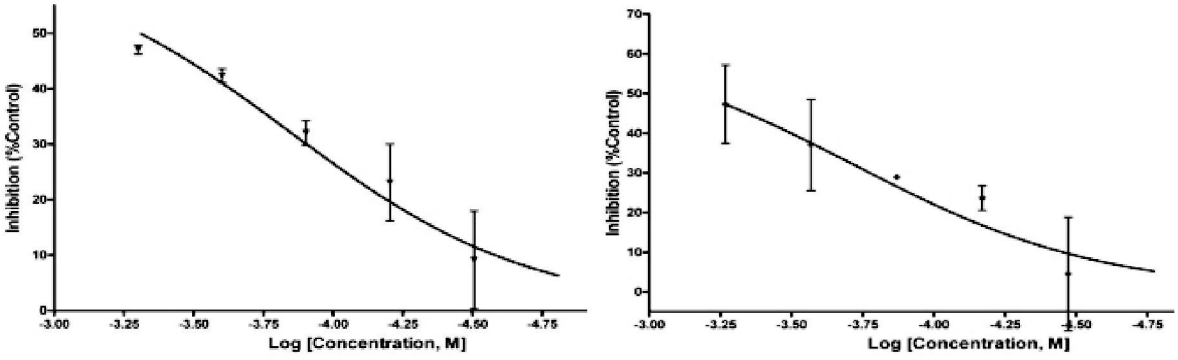
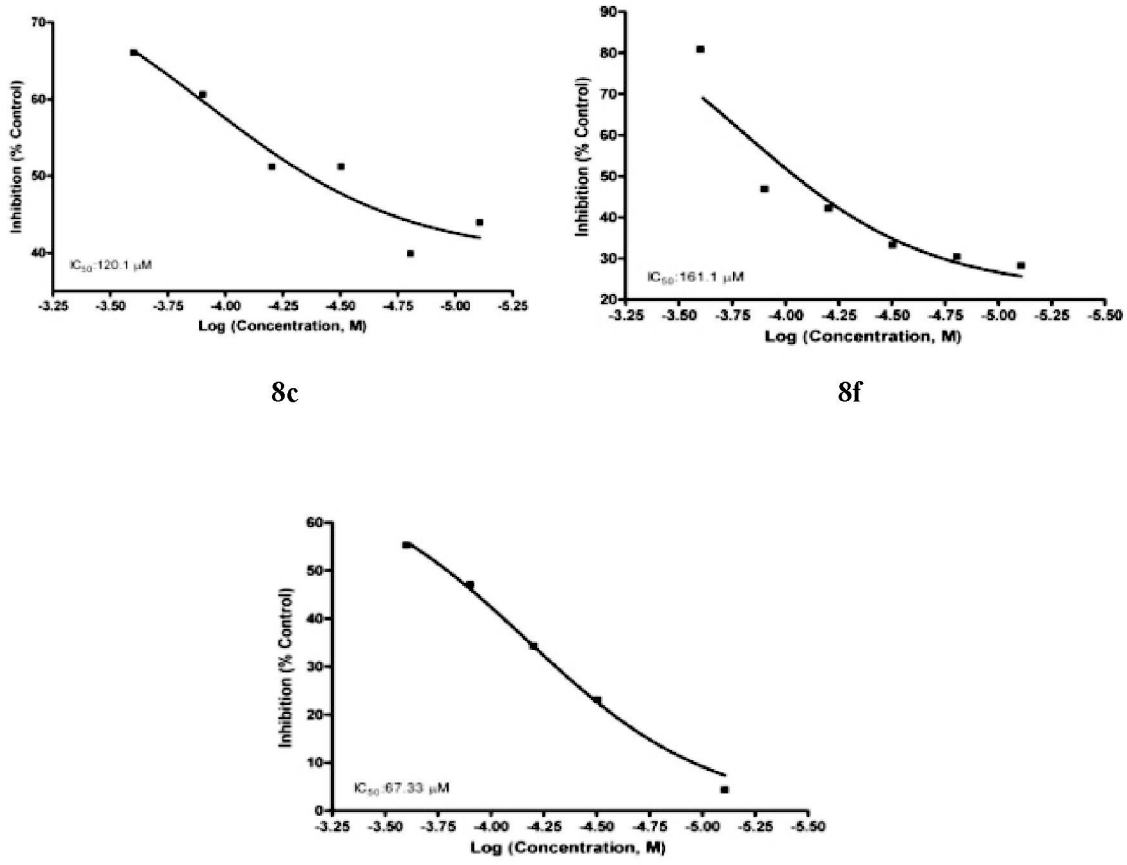


Figure 6. Lyn kinase inhibition of compounds 8g and 8h. Data shown here is the prescreening result of compounds at two different concentrations, each in duplicate.

Table 1. The IC_{50} (μM) values of the Compounds 8c, 8f, 8g, and 8h for c-Src (19), Fyn, Lyn and Hck kinases and GST are given.

Comp. No	c-Src	Fyn	Hck	Lyn	GST
8c	4.69	249.2	No Inhibition	431.3	120.1
8f	74.79	130.6	> 1mM	No Inhibition	161.1
8g	75.06	326.5	55.41	90.43	67.33
8h	84.23	75.46	75.49	187.4	272.2

Among these, the best GST inhibitior is found as compound 8g followed by compound 8c (Figure 7). The compounds 8c and 8g exhibited GST inhibiton with IC_{50}s of 120.1, and 67.33 μM concentrations, respectively, and are also reported as c-src inhibitors. Therefore these compounds can be considered as dual inhibitor. None of the compounds showed a reasonable Hck inhibition under the experimental conditions. Further studies on new potent Src and GST inhibitors will provide new aspects for the discovery and development of selective and drug resistant compounds for the treatment of cancer. Since very small number of compounds were tested for the determination of their selectivity profile of SFK enzymes and dual actions, our future aim is going to find more potent inhibitor against both SFK and GST enzymes.



8c **8f**

8g

Figure 7. GST inhibition of compounds **8c**, **8f**, and **8g.**

ACKNOWLEDGEMENTS

This project is supported by a fund from Ankara University (BAP, 09B3336002).

REFERENCES

1. Biscardi JS, Tice DA, Parsons SJ, c-Src, receptor tyrosine kinases, and human cancer, Adv Cancer Res 76, 61-119, 1999.
2. Martin GS, The road to Src, Oncogene 23, 7910-7917, 2004.
3. Lowell CA, Soriano P, Knockouts of Src-family kinases: stiff bones, wimpy T cells, and bad memories, Genes Dev 10, 1845-1857, 1996.
4. Bjorge JD, Jakymiw A, Fujita DJ, Selected glimpses into the activation and function of Src kinase, Oncogene 19, 5620-5635, 2000.
5. Ingley E, Src family kinases: Regulation of their activities, levels and identification of new pathways, Biochem et Biophys Acta 1784, 56-65, 2008.
6. Brown MT, Cooper JA, Regulation, substrates and functions of src, Biochim Biophys Acta 1287, 121–149, 1996.
7. Rucci N, Susa M, Teti A, Inhibition of protein kinase c-Src as a therapeutic approach for cancer and bone metastases, Anti-Cancer Agents in Med Chem 8, 342-349, 2008.
8. Schwartzberg PL, The many faces of Src: multiple functions of a prototypical tyrosine kinase, Oncogene 17, 1463-1468, 1998.
9. Thomas SM, and Brugge JS, Cellular functions regulated by Src family kinases, Annu Rev Cell Dev Biol 13, 513-609, 1997.
10. Frame MC, Src in cancer: deregulation and consequences for cell behaviour, Biochim Biophys Acta 1602, 114-130, 2002.
11. Warmuth M, Damoiseaux R, Liu Y, Fabbro D, Gray N, Src family kinases: potential targets for the treatment of human cancer and leukemia, Curr Pharm Des 9, 2043-2059, 2003.
12. Belsches-Jablonski AP, Demory ML, Parsons JT, Parsons SJ, The src pathway as a therapeutic strategy, Drug Discovery Today: Therapeutic Strategies 2, 313-321, 2005.
13. Alvarez RH, Kantarjian HM, Cortes JE, The role of Src in solid and hematologic malignancies: development of new-generation Src inhibitors, Cancer 107, 1918-1929, 2006.
14. Hayes JD, Pulford DJ, The glutathione S-transferase supergene family: regulation of GST and the contribution of the isoenzymes to cancer chemoprotection and drug resistance, Crit Rev Biochem Mol Biol 30, 445-600, 1995.
15. Di Pietro G, Magno L, Rios-Santos F, Glutathione S-transferases: an overview in cancer research, Expert Opin Drug Metab Toxicol 6, 153-170, 2010.
16. Baez S, Segura-Aguilar J, Widersten M, Johansson AS, Mannervik B, Glutathione transferases catalyse the detoxication of oxidized metabolites (o-quinones) of catecholamines and may serve as an antioxidant system preventing degenerative cellular processes, Biochem J 324, 25-28, 1997.
17. Scotlandi K, Remondini D, Castellani G, Manara MC, Nardi F, Cantiani L, Francesconi M, Mercuri M, Caccuri AM, Serra M, Knuutila S, Picci P, Overcoming resistance to conventional drugs in Ewing sarcoma and identification of molecular predictors of outcome, J Clin Oncol 27, 2209-2216, 2009.
18. Hayeshi R, Chinyanga F, Chengedza S, and Mukanganyama S, Inhibition of human glutathione transferases by multidrug resistance chemomodulators in vitro, J Enzyme Inhib Med Chem 21, 581-587, 2006.
19. Kılıç Z, İşgör YG, Ölgen S, Synthesis and $pp60^{c\text{-}Src}$ tyrosine kinase inhibitory activities of novel indole-3-imine and amine derivatives substituted at N1 and C5, Arch Pharm 342, 333-343, 2009.
20. Habig WH, Jakoby WB, Assays for differentiation of glutathione S-transferases, Methods Enzymol 77, 398-405, 1981.
21. Juronen E, Tasa G, Uusküla-Pooga M, Mikelsaar AV, Purification, characterization and tissue distribution of human class theta glutathione S-transferase T1-1, Biochem Mol Biol Int 39, 21-29, 1996.

22. Gusson F, Carletti M, Albo AG, Dacasto M, Nebbia C, Comparison of hydrolytic and conjugative biotransformation pathways in horse, cattle, pig, broiler chick, rabbit and rat liver subcellullar fractions, Vet Res Commun 30, 271-283, 2006.
23. Technical Bulletin, Profluor Src-Family Kinase Assay, Instruction for use of products V1270 and V1271, Promega Corporation, Printed in USA. 6/2009.

Received: 27.06.2011
Accepted: 14.07.2011