Med Chem Res (2014) 23:1225–1233
DOI 10.1007/s00044-013-0729-7

MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Cytotoxic activity assessment and c-Src tyrosine kinase docking simulation of thieno[2,3-*b*] pyridine-based derivatives

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Received: 11 June 2013 / Accepted: 14 August 2013 / Published online: 29 August 2013

Abstract Thienopyridine derivatives possess various promising biological properties and particularly cytotoxic effect. In vitro cytotoxic activities of some thienopyridine analogous were evaluated by MTT reduction assay in three human cancer cell lines (HL-60, MCF-7, and LS-180). The compounds showed a wide range of cytotoxic activities and their IC_{50} values ranged from 0.2 to 100 μM and above. Compound **4e** was the most potent derivative and **4i** showed good cytotoxic activity against all three cell lines (IC_{50} <20 μM). Docking simulation of thienopyridine derivatives was implemented on c-Src tyrosine kinase involved in tumor progression and metastases. Results showed that these compounds might potentially bind to the key amino acid Thr339 in the c-Src tyrosine kinase active site. Ligand efficiency (LE) values calculated by using free binding energies obtained from experimental data were predicted by the docking study. Also, experimental and predicted LEs were in good agreement. Based on the LE indices and other findings, some of the thienopyridine derivatives might be efficient candidates for further development as anticancer agents.

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Keywords Thienopyridine · Cytotoxic activity · Docking simulation · Ligand efficiency

Introduction

Cancer is amongst the most serious health threats in the world. Therefore, researchers have been investigating various clinical approaches against cancer (Azizmohammadi *et al*., 2013). Although many chemotherapeutic agents have been developed for management of cancer, this disease still causes morbidity and mortality. Therefore, there is an urgent need for novel anticancer agents aimed at important biological targets for cancer treatment (Liu *et al*., 2012; Penthala *et al*., 2010; Azizmohammadi *et al*., 2013).

Several thienopyridine derivatives have been developed as cytotoxic and antitumoral agents (Abreu *et al*., 2011; Boschelli *et al*., 2005; Pevet *et al*., 2011; Queiroz *et al*., 2010; Zeng *et al*., 2010). Biological activities exhibited by thienopyridine derivatives include antimicrobial (Abdel-Rahman *et al*., 2003), antiviral (Schnute *et al*., 2005), antiallergic (Youssefyeh *et al*., 1984), anti-inflammatory (Morwick *et al*., 2006), and modulation of muscarinic acetylcholine receptors (mAChRs) (Shirey *et al*., 2007). They also possess other useful pharmacological properties such as inhibiting the mitogen-activated protein kinase enzymes (Trujillo, 2011) and promoter of bone formation (Saito *et al*., 2013). Thienopyridines are one of the privileged scaffolds that were identified and developed as a novel class of Src kinase inhibitors (Boschelli *et al*., 2004, 2005; Pevet *et al*., 2011; Atatreh *et al*., 2008).

Src is a non-receptor tyrosine kinase, which is present in cytoplasm and belongs to the a structurally related kinase named Src family of kinases (SFKs) (Huang *et al*., 2010; Lee *et al*., 2009; Noronha *et al*., 2006). Src deregulation is

associated with metastases, tumor progression, neovascularization, and poor prognosis. Aberrant Src kinase activity has been linked with metastatic bone disease occurring in many advanced solid tumor cancers. Synergistic activity of Src kinase inhibitors with hormonal and cytotoxic agents has been proposed in preclinical studies (Pengetnze *et al*., 2003; Shah and Rowan, 2005).

Binding of c-Src to focal adhesion kinase (FAK) causes activation of c-Src/FAK signaling cascade. c-Src/FAK complex could induce tumor growth and metastasis in many tumors. Furthermore, stimulation of Src results in activation of the signal transducer and activator of transcription (STAT3). STAT3 promotes production of VEGF, which has a role in angiogenesis, invasion, and metastasis (Src/STAT3/VEGF pathway) (Guarino, 2010; Mitra and Schlaepfer, 2006).

Src is regarded as an important target for cancer therapy and several Src kinase inhibitors have been reported (Lee *et al*., 2009). Pevet et al. (2011) evaluate inhibitory activity of thienopyridine derivatives on the phosphorylation of endogenous Src substrates including FAK and STAT3 in two carcinoma cells by western blot assays. They demonstrated that thienopyridine derivatives could inhibit Src tyrosine kinase activity in vitro and have cellular c-Src inhibitory activity in human carcinoma cells.

For the synthesis of thienopyridine scaffold, Thorpe isomerization of substituted 2-alkylthio-3-cyanopyridines is of considerable interest (Litvinov *et al*., 2005). The benefits of this method are that the starting 1,2-dihydro-2-thioxopyridine-3-carbonitriles (**1**) are available, diverse 1,2-dihydro-2-thioxopyridine-3-carbonitriles can be used, one-pot procedures can be utilized, and the yields of final products are high (Fig. 1).

In this contribution, we aimed at determining cytotoxic effects and molecular modeling of some new thienopyridine derivatives. The synthesis and structural characterization of the thienopyridine compounds (**4a–j**) were reported previously (Salarian *et al*., 2012).

Based on the previous studies, Src deregulation was particularly observed in colon and breast cancers (Pevet *et al*., 2011), therefore MCF-7 (human breast adenocarcinoma) and LS-180 (human colon adenocarcinoma) cell lines were chosen for this study. Docking simulation was implemented to investigate binding pattern and pose of theino [2,3-*b*] pyridine derivatives in the binding site of c-Src tyrosine kinase.

Experimental

Cytotoxicity section

Reagents and chemicals

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Sigma (Saint Louis, MO, USA), and penicillin/streptomycin was purchased from Invitrogen (San Diego, CA, USA). Fetal bovine serum (FBS), phosphate-buffered saline (PBS), RPMI 1640, trypan blue, and trypsin were purchased from Biosera (Ringmer, UK). Cisplatin and doxorubicin were obtained from EBEWE Pharma (Unterach, Austria). Dimethyl sulfoxide was obtained from Merck (Darmstadt Germany).

Cell lines and culture

Three cell lines were used in this study including HL-60 (acute promyelocytic leukaemia), MCF-7 and LS-180. All cell lines were purchased from the National Cell Bank of Iran, Pasteur Institute, Tehran, Iran. Cells were maintained at 37 °C in humidified air containing 5 % CO_2. All cell lines were maintained in RPMI 1640 supplemented with 10 % FBS, and 100 U/ml penicillin-G and 100 mg/ml streptomycin. HL-60 cell was grown in suspension, while MCF-7 and LS-180 cells were grown in monolayer cultures.

Cell viability assay

Cytotoxic activities of some synthetic analogous were estimated by MTT reduction assay (Mosmann, 1983; Miri *et al*., 2011). HL-60, MCF-7, and LS-180 cells were plated in 96-well micro-plates at densities of 40000, 30000, and 50000 cells/ml, respectively (100 µl per well). After overnight incubation at 37 °C, 50 µl of the growth medium replaced with the medium treated with 3–4 different concentrations of synthetic compounds dissolved in DMSO. Plates with HL-60 cells were centrifuged before this procedure. Concentration of DMSO in the wells was lower than 0.5 %. Incubation was continued for 72 h and at the end of the incubation time the medium replaced with MTT solution in phosphate-buffered saline at a final concentration of 0.5 mg ml^{-1} and plates were incubated for another 4 h at 37 °C. The formazan crystals dissolved in 200 µl of

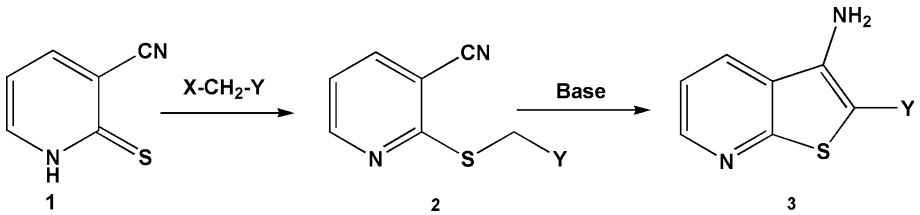
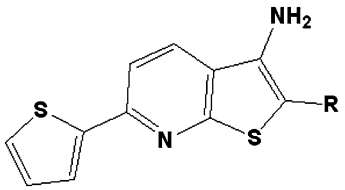
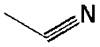
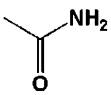
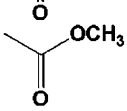
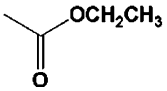
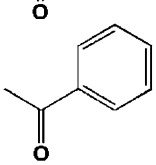
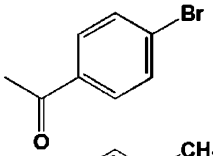
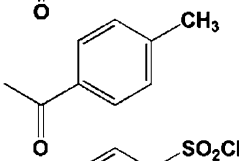
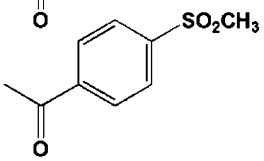
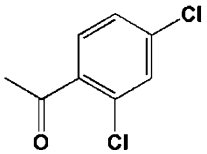
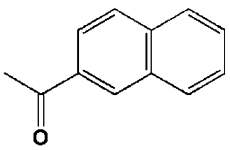


Fig. 1 Thorpe isomerization of 1,2-dihydro-2-thioxopyridine-3-carbonitrile, X is leaving group, Y is EWG

Table 1 Cytotoxic activity of some thienopyridine derivatives



Compound	*R*	IC_{50} (μM)[a]		
		HL-60	MCF-7	LS-180
4a		>100	>100	>100
4b		21.3 ± 3.5[a]	26.2 ± 9.5	20.9 ± 6.9
4c		>100	>100	>100
4d		>100	>100	>100
4e		0.2 ± 0.0	1.3 ± 0.1	0.6 ± 0.1
4f		62.2 ± 6.0	31.5 ± 3.4	26.3 ± 4.4
4g		25.6 ± 2.1	25.1 ± 1.0	20.8 ± 6.5
4h		12.2 ± 1.5	>100	65.2 ± 9.9
4i		15.5 ± 0.5	13.2 ± 1.0	14.0 ± 1.7
4j		39.6 ± 1.1	31.0 ± 5	37.6 ± 8.5
Cisplatin		2.1 ± 0.2	15.2 ± 2.2	37.6 ± 1.3
Doxorubicin		0.01 ± 0.00	0.10 ± 0.02	0.02 ± 0.01

[a] Values show the average of three to five experiments ±SEM

Table 2 Correlation coefficient (R^2) between IC_{50} values in three cell lines

	HL-60	LS-180	MCF-7
HL-60	1		
LS-180	0.728	1	
MCF-7	0.511	0.926	1

DMSO. The optical density was recorded at 570 nm with background correction at 655 nm using a Bio-Rad microplate reader (Model 680). Blank wells contained same concentrations of synthetic compounds without MTT solution used for background correction. The percentage of viability compared to control wells (untreated wells) was calculated for each concentration of the compound and IC_{50} values were calculated with the software CurveExpert version 1.34 for Windows. Each experiment was repeated three to five times and each of them was in triplicate. Data are presented as mean ± SEM.

Docking study

Docking studies were performed by using the program AutoDock 4.2. X-ray crystal structure of c-Src tyrosine kinase receptor in the active form was retrieved from Brookhaven protein data bank with PDB entry 1y57, resolution 1.91 Å (http://www.rcsb.org). All the pre-processing steps for receptor structure were done by using WHAT IF server (European Molecular Laboratory Heidelberg, Germany) and AutoDock Tools 1.5.4 program (ADT). The method of ligand optimization was AM1 using Polak-Ribiere (conjugate gradient) algorithm with final condition as RMS gradient of 0.1 (Kcal/Å mol).

A grid of 60, 60, and 60 points in *x*-, *y*-, and *z* directions, with grid spacing of 0.375 Å were centered on the binding site of c-Src tyrosine kinase and calculated by AutoGrid. The search algorithm in this study was Lamarckian Genetic Algorithm (LGA) (Morris *et al*., 2009). For each 100-independent runs, a maximum number of 2,500,000 energy evaluations; 27,000 maximum generations; a gene mutation and a crossover rates of 0.02; and 0.8, respectively, were used.

A root-mean-square (RMS) tolerance of 2 Å was considered for clustering the results. The conformation with minimum predicted-binding energy was considered as the best docking result. Generation of schematic 2D ligand–receptor interaction maps was performed using LIGPLOT (Wallace *et al*., 1995).

Results and discussion

Cytotoxic activity analysis

The cytotoxic activities of compounds **4a–j** against three cell lines (HL-60, LS180 and MCF-7) in comparison with cisplatin and doxorubicin are shown in Table 1. Evaluated compounds showed a wide range of cytotoxic activity. Their IC_{50} ranged from 0.2 to 100 μM and above. The promising compound of these series was **4e** containing phenyl substituent that showed significant potency against HL-60, MCF-7, and LS-180 (IC50: 0.2, 1.3, and 0.6 μM, respectively). It was more potent than cisplatin against all tumor cell lines. Also, **6i**-containing 2,4-dichlorobenzoyl substituent showed considerable cytotoxic activity against HL-60, MCF-7, and LS-180 cells with IC_{50} values of 15.5, 13.2, and 14.0 μM, respectively. Three compounds **4a**, **4c**, and **4d** containing cyano, methyl carbonoyl, and ethyl carbonoyl, respectively, were the weakest cytotoxic agents showing no activity at concentrations lower than 100 μM.

Table 3 AutoDock-based binding free energies (ΔG_b), inhibition constants (K_i) along with experimental-binding free energies (ΔG_{exp}) of thienopyridines in the c-Src tyrosine kinase (PDB ID: 1Y57)

Compounds	Estimated k_i (μM)	Estimated ΔG_b (kcal/mol)	ΔG^a_{exp}		
			HL-60	HL-60	LS-180
4a	12.18	−6.70	–	–	–
4b	3.58	−7.43	−6.37	−6.24	−6.38
4c	22.55	−6.34	–	–	–
4d	9.44	−6.86	–	–	–
4e	0.358	−8.79	−9.05	−8.02	−8.52
4f	1.70	−7.87	−5.73	−6.13	−6.24
4g	0.456	−8.65	−6.26	−6.27	−6.38
4h	1.02	−8.17	−6.70	−5.45	−5.70
4i	0.307	−8.88	−6.55	−6.65	−6.61
4j	0.496	−8.6	−6.00	−6.14	−6.03

[a] $\Delta G_{exp} = -RTLnIC_{50}$

Compound **4h** bearing methyl sulfonyl benzoyl moiety showed significant cytotoxicity against HL-60 and had fairly selective effect in this cell line.

The structure–activity relationship (SAR) assessment indicated that compounds containing aryl substituent were more active than compounds bearing other moieties.

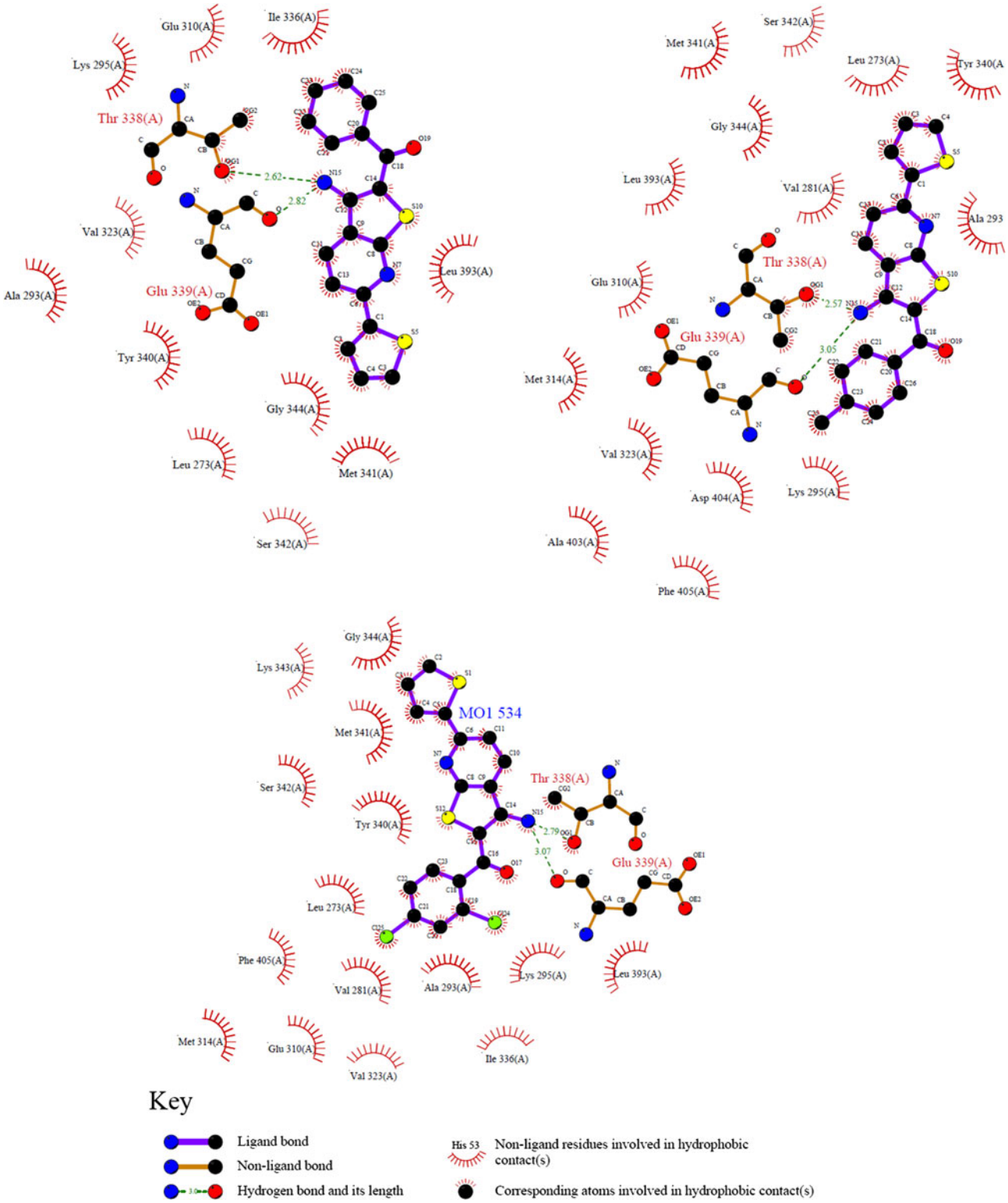


Fig. 2 2D scheme interaction between **4i**, **4e**, **4g** and c-Src tyrosine kinase active site generated by LIGPLOT, PDB ID:1Y57

Exceptionally, **4b** bearing carbamoyl moiety showed considerable activity against all three cell lines. By comparing the activities of compounds containing substituted aryl moieties **4f–j** with their un-substituted counterpart **4e**, it was revealed that introduction of electron withdrawing groups (methyl sulfonyl) and electron donating groups (methyl) on phenyl ring significantly decrease the cytotoxic potency in all cell lines. Comparison of compounds having substituted aryl moieties **4f–j** demonstrated that introduction of 2,4-dichloro substituent in aryl moiety **4i** can significantly improve the cytotoxic potency against all tumor cell lines.

Correlation of cytotoxic data

The correlation between cytotoxic activities in three cell lines showed interesting results (Table 2). IC_{50} values in LS-180 and MCF-7 cells showed strong correlation with each other ($R^2 = 0.926$, data not shown). This correlation may indicate possible similar mechanisms of cytotoxicity on these cancer cell lines. This finding might increase the possibility of interaction between thienopyridines and Src tyrosine kinase because Src deregulation have been particularly observed in colon and breast cancers. It was also revealed that IC_{50} values in HL-60 data did not show good correlation with that of LS-180 and MCF-7 data.

Molecular docking

All thienopyridine derivatives were docked into the active site of c-Src tyrosine kinase. The outputs of the docking study are summarized in Table 3.

As shown in Table 3, compounds exhibiting the lowest binding free energies (the highest binding affinities) into the c-Src tyrosine kinase are: **4i** (ΔG_b: −8.88 kcal/mol), **4e** (ΔG_b: −8.79 kcal/mol) and **4g** (ΔG_b: −8.65 kcal/mol). As depicted in Fig. 2, three top ranked compounds exhibited similar hydrogen bonds to Thr 338 and Glu 339. Moreover, these compounds showed similar hydrophobic interactions with Leu 273, Ala 293, Lys 295, Glu 310, Val 323, Tyr 340, Met 341, Ser 342, Gly 344, and Leu 393. Based on the H-bonds and hydrophobic interactions, **4i**, **4e**, and **4g** exhibited similar binding patterns and pose in the active site of c-Src tyrosine kinase. Molecular docking study predicted that compounds **4i**, **4e**, and **4g** might be biologically good antitumor compounds that were confirmed by the experimental cytotoxicity data. The characteristics of possible key H-bonds between theinopyridines and c-Src tyrosine kinase were summarized in Table 4. Based on the previous binding models, Thr 339 is one of the key active site residues playing an important role for inhibition of tyrosine kinase (Huang *et al*., 2010; Thaimattam *et al*., 2005). Compounds **4i**, **4e**, and **4g** exhibited a similar H-bond pattern between their 3-amino moiety and the side chain OH group of Thr339. Compounds **4a**, **4c**, and **4d** showed the lowest binding affinities with ΔG_b: −6.70, −6.34, and −6.86 (kcal/mol), respectively. Regarding the results, molecular docking study predicted weak biological antitumor activities for them that are in agreement with our experimental results. Figure 2 illustrate 2D scheme of interaction between docked **4i**, **4e**, and **4g** compounds and active site of c-Src tyrosine kinase.

Ligand efficiency indices

Ligand efficiency (LE) is a useful parameter that guides us to optimized drug discovery process (Abad-Zapatero and Metz, 2005). The LE definition is the binding energy of ligand per heavy atoms (i.e., non-hydrogen atoms) (Hopkins *et al*., 2004). LE could be used as a filter for all synthesized molecules or fragments with the aim of developing efficient biologically active structures. LE concept explain that molecules with a given potency and fewer heavy atoms are considered more efficient (Abad-Zapatero and Metz, 2005; Azizian *et al*., 2012). In this study, LE were calculated using free energy of ligand binding obtained from experimental data and predicted by docking study. Possible correlation between experimental and predicted LEs was evaluated. The correlation coefficient between experimental LEs on HL-60, MCF-7, and

Table 4 Possible key hydrogen bonds for three top ranked thienopyridines in the active site of c-Src tyrosine kinase obtained from docking study

Compounds	H-bonds between atom of compounds and amino acid residues			
	Atom of comp.	Amino acid	Distance (Å)	Angle (°)
4i	$-N_{15}H$[a]	OH of Thr339	2.17	18.24
		O of Glu339	2.24	12.2
4e	$-N_{15}H$	OG1 of Thr339	2.07	20.36
		O of Glu339	1.98	12.75
4g	$-N_{15}H$	OG1 of Thr339	1.91	19.31
		O of Glu339	2.26	13.2

[a] For numbering, readers are referred to Fig. 1

Table 5 Experimental and predicted ligand efficiency (LEs)

Compounds	Experimental LE[a,b] (kcal/mol atom)			Predicted LE[c] (kcal/mol atom)
	LE of HL-60	LE of MCF-7	LE of LS-180	
4a	–	–	–	–
4b	0.353	0.347	0.354	0.412
4c	–	–	–	–
4d	–	–	–	–
4e	0.393	0.348	0.370	0.382
4f	0.239	0.255	0.26	0.328
4g	0.260	0.261	0.266	0.360
4h	0.248	0.201	0.211	0.302
4i	0.262	0.266	0.264	0.355
4j	0.222	0.227	0.223	0.318

[a] Experimental LE = $\Delta G_{exp}/N$ (N is the number of non-hydrogen atoms)

[b] The least optimum amount for LE is 0.3

[c] Predicted LE = $\Delta G_b/N$

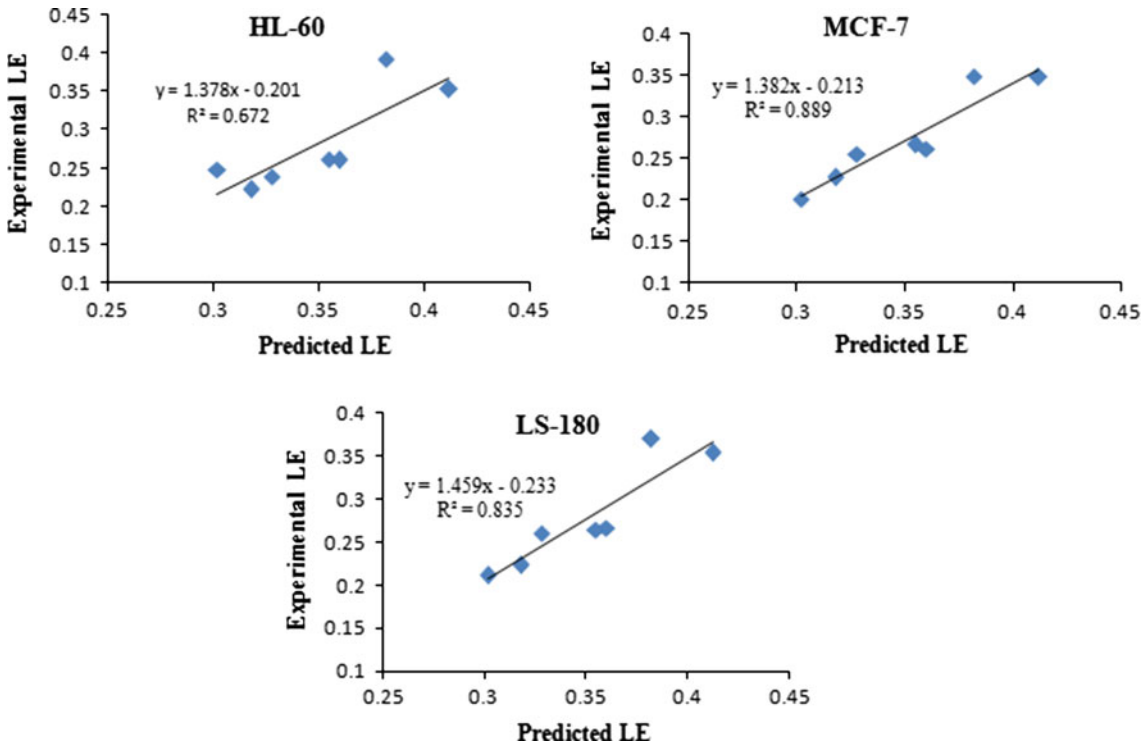


Fig. 3 The correlation coefficient (R^2) between experimental LE on HL-60, MCF-7, and LS-180 tumor cell lines and predicted LE

LS-180 tumor cell lines and predicted LEs based on docking study were 0.672, 0.889, and 0.835, respectively. These results indicated that our docking study was successful in predicting LE of these compounds. Compounds **4e** and **4b** showed good cytotoxic activity and had reasonable LE. Therefore, they can be regarded as among promising candidates for further SAR developments (Table 5; Fig. 3).

Conclusion

In this study, a series of thienopyridine derivatives were evaluated in vitro for their cytotoxic activity on three human cancer cell lines (HL-60, MCF-7, and LS-180). Src deregulation particularly occurs at colon and breast cancers therefore these cell lines were chosen for this study. Some of the studied compounds revealed moderate-to-good

cytotoxic activities, among which, best cytotoxic activities were exhibited by compounds **4e** and **4i**. In addition to good cytotoxic activities of **4e** and **4i**, they are two top ranked compounds from docking study. These two compounds are also efficient with reasonable predicted LE (0.382 and 0.355, respectively). Experimentally calculated and predicted LE was in good agreements. These results indicated that docking study might predict efficiency of these compounds. Docking simulation showed that these compounds might potentially bind to the key amino acid Thr339 in c-Src tyrosine kinase active site. Cytotoxic activities in LS-180 and MCF-7 tumor cell lines exhibited strong correlation ($R^2 = 0.926$). These may indicate similar cytotoxicity mechanism and illustrates the possibility of interaction between thienopyridine and c-Src tyrosine kinase in colon and breast cancers. Our findings showed that thienopyridines are hopeful scaffolds for future bioactive molecular design as promising cytotoxic agents. Moreover, c-Src tyrosine kinase might be a potential target for further investigation based on thienopyridine scaffolds.

Acknowledgments Financial supports of this project by AJA University of Medical Sciences are acknowledged. We gratefully acknowledge the financial support of the Shiraz University of Medical Sciences, vice-chancellor of research.

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