A Study on the Interaction between p60$^{c\text{-}Src}$ Receptor Tyrosine Kinase and Arylcarboxylic and Arylacetic Acid Derivatives Based on Docking Modes and *in Vitro* Activity

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The fundamental role that receptor tyrosine kinases play in cancer and other proliferative diseases has provided the impetus for an extensive effort on the part of both academic and pharmaceutical laboratories to develop highly specific inhibitors. In this study, inhibitory activity of previously synthesized arylacetic and arylcarboxylic acid derivatives were examined against substrate of tyrosine kinase. It can be assumed that the activity of compounds becomes higher when the $-CH_2$ linkage exist between aromatic ring and the amide group of the side chain. In addition, when the R_1 and R_2 substitutents are methyl group in both series, the higher activity observed. The data obtained from docking study (DOCK4.0) indicated that compounds 2, 4, 7, 8, 11 render satisfactory interaction with the active site of enzyme, Lys295 of p60$^{c\text{-}Src}$ tyrosine kinase. Comparison of this interaction and the evaluation of biological data showed that compound 4 is the most active among the entire derivatives.

Key words arylacetic acid; arylcarboxylic acid; tyrosine kinase; molecular docking

Cancer is one of the first leading causes of death throughout the world. Therefore, many different types of study are focused on developing new compounds, which show anticancer activity by different way of actions. In recent years, designing of new compounds with the inhibitory activity of angiogenesis is considered hoping that this would be a promising approach to cancer chemotherapy. Tumor angiogenesis is the proliferation of a network of blood vessels that penetrates into cancerous growths, supplying nutrients and oxygen and removing waste products. Very recent studies showed that angiogenesis can induce the formation of cancerous tumors and the inhibition of angiogenesis can inhibit growing and spreading of tumors. The importance of protein tyrosine kinases (PTKs) in signal transduction and proliferative disorders associated with a variety of human cancers makes agents attractive therapeutic targets and pharmacological probes, which modulate the activity of PTKs.[1—8] In this respect, it is found that many common rings, including different heterocyclic and simplified aromatic structures were important as PTKs inhibitors. As a result of the relationship found inflammation and angiogenesis, some anti-inflammatory agents can be delineated to serve as a basis for the design of PTKs.[4,5,7,9—11] Therefore, it was of interest to investigate the ability of arylacetic and arylcarboxylic acid derivatives (Tables 1, 2) for their PTKs inhibitory activity through docking modes and *in vitro* activity tests. In order to identify the interaction between synthesized compounds and the enzyme active site, molecular modeling studies have been applied.[12] The data obtained from DOCK 4.0 program showed that some of our compounds have good interactions with the enzyme active site and led us to test their tyrosine kinase inhibitory activity by ELISA method.[13]

MATERIALS AND METHODS

Materials The docking experiments as well as receptor and ligand preparations were performed on SGI Indigo Extreme (R4400) workstation. Insight II software (MSI) and DOCK 4.0 were used as molecular modeling software. Universal tyrosine assay kit (Takara Universal Co., Japan) was used to test compounds against p60$^{c\text{-}Src}$ tyrosine kinase. *N*,*N*-Dimethylformamide, 1 N sulfuric acid, and .05% tween in PBS (phosphate-buffered saline) were purchased from Wako Pure Chemical Co., Ltd. Osaka, Japan. 2-Mercaptoethanol was supplied from ICN Biomedicals Inc. (Japan). An incubator (Masuda SA-30, Japan) and micro TAITA plate reader (BioRad 550, Japan) were used for tyrosine kinase assay.

Tyrosine Kinase Assay The phosphorylation assays were performed at 37 °C in a final volume of 40 μl of tyrosine kinase. The concentrations of PTKs used to construct calibration curve were as follows: 600, 500, 341, 200, 100×10^{-7} units/μl for p60$^{c\text{-}Src}$ PTK. The unit of $500\times10^{-7}/\mu$l was used for each inhibitor. Phosphorylation reactions were initiated with the addition of 40 mM ATP (10 μl) into each vessel and then the plate was incubated at 37 °C for 30 min. After the completion of reaction, liquid was decanted and the vessels were washed with tween-PBS for 4 times. One hundred microliters of blocking solution was added to the vessels and incubated at 37 °C for 30 min. After washing the plate with tween-PBS, 50 μl of anti-phosphotyrosine was added to the vessels and incubated at 37 °C for 30 min. The reaction liquid was decanted and the remaining solution was removed by rinsing with tween-PBS for 4 times. One hundred microliters HRP coloring agents was added and incubated at 37 °C for 15 min. Reaction is terminated by the addition of 100 μl/well 1 N sulfuric acid. The absorbance of reaction was measured at 450 nm in microplate reader.

Docking Study The DOCK 4.0 program,[12] was utilized for the identification of specific inhibition properties of arylcarboxylic and arylacetic acid derivatives on p60$^{c\text{-}Src}$ tyrosine kinase enzyme. The docking experiments as well as receptor and ligand preparations were performed on SGI Indigo Extreme (R4400) workstation. Insight II software (MSI)[14] was used for drawing compounds. To attach hydrogen atoms,

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molecules are converted to the Sybyl (Tripos) mol2 files. PP1 was taken as the ligand and has been docked manually into the active site. Empirical partial atomic charges were taken from the CVFF force field with the assistance of Insight II software. The enzyme, $p60^{c\text{-}Src}$ tyrosine kinase, taken out from PDB (Protein Data Bank), was placed into the Insight II on which docking with inhibitors were conducted.

The DOCK program is specifically designed for the identification of small molecule inhibitors which are complementary to a targeted surface area or an active site.[14] DOCK first generates a negative image of the ligand binding site with a set of overlapping spheres whose centers become the potential locations for the ligand atoms rotation. To rank each potential inhibitor, a pre-calculated contact-scoring grid, based on distance between potential inhibitor atoms and target area atoms, and a force-field-scoring grid, based on molecular mechanics interaction energies consisting of Van der Waals and electrostatic components were generated. The resulting output file for each screening, based on distance or force field grids, contains the highest scoring compounds ranked in order of their scores.

Based on the information on the pocket, an inhibitor was placed in the pocket manually. DOCK 4.0, filled the pocket with spheres, moved an inhibitor to the center of each sphere, and rotated it to score the docking energy. This procedure of transforming and rotating the inhibitor was repeated at each sphere center.

Synthesized compounds were drawn on the SGI (O2) workstation by using Insight II. The charge was assigned on the drawn compound which was optimized by Discover.

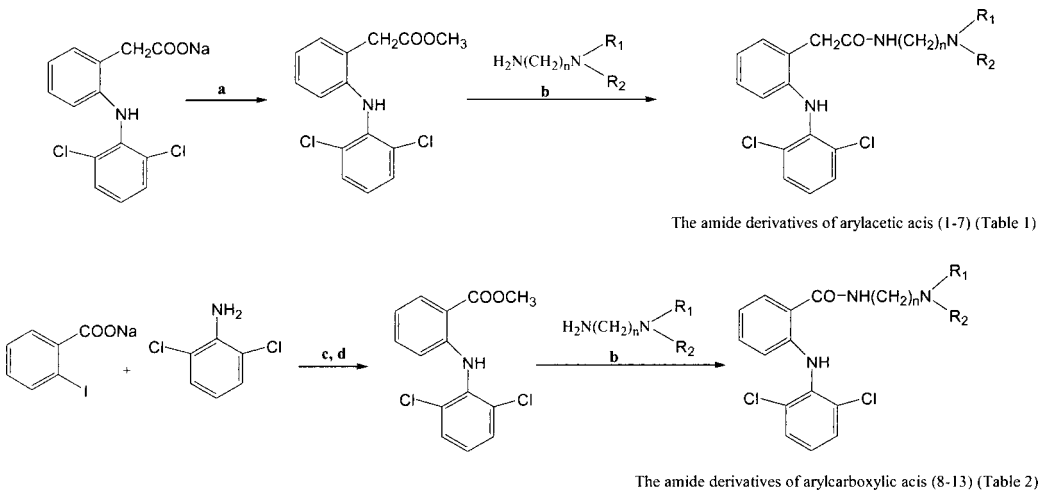
RMSD is computed and expressed in Å, and it is a structural comparison of two molecules in terms of distance. Assume that the structure is defined in terms of the Cartesian coordinates of the atoms and represented by an $n\times3$ coordinate matrix, where n is the number of atoms in the molecule. Structure translation can then be done *via* adding a translation vector to each row of the coordinate matrix, and structure rotation *via* multiplying the coordinate matrix by a rotation matrix. Let X and Y be the coordinate matrixes of two structures after they are translated so that their centers of geometry coincide.

After docking of the proposed compounds against $p60^{c\text{-}Src}$ tyrosine kinase, the top 25 docking results, which were arranged in order with the one with lowest energy first, were examined. The hydrogen bonds between compounds and enzyme were evaluated by using Insight II software. The compound with the highest number of hydrogen bond with the lowest binding energy was noted for evaluating inhibition capacity and selectivity for the enzyme active site. The compound which shows the lowest binding energy with the greatest number of hydrogen bonds, will be docked more firmly and inhibit the enzyme more strongly than the other compounds.

RESULTS AND DISCUSSION

The synthesis of arylcarboxylic and arylacetic acid derivatives were previously reported by Nebioglu *et al.*[15] Briefly, the amide derivatives of arylacetic acid derivatives were synthesized (Chart 1) from the methyl ester of diclofenac by reacting them with several substituted diamine derivatives. For the synthesis of arylcarboxylic acid amides, sodium *o*-iodo benzoate was used as a starting compound. Condensation of the 2,6-dichloroaniline with sodium *o*-iodo benzoate gave the 2-[(2,6-dichlorophenyl)amino]benzoic acid. The methyl ester of acid was prepared and transformed into amide, reacting with several substituted diamine derivatives.

DOCK 4.0 program,[12] was utilized for the identification of specific inhibition properties of arylcarboxylic and arylacetic acid derivatives on $p60^{c\text{-}Src}$ tyrosine kinase enzyme. The DOCK 4.0 program can be summarized as a search for geometrically allowed ligand-binding modes using several steps that include: describing the ligand and receptor cavity as sets of spheres, matching the sphere sets, orienting the ligand, and scoring the orientation.[16,17] It was stated that in some cases DOCK 4.0 program offers a better correlation with respect not only to orientation of molecules inside the cavity but also to docking scores.[18] It is known that compounds, which dock better than the others, have potential better binding capacity to the enzyme active site.



The amide derivatives of arylacetic acis (1-7) (Table 1)

The amide derivatives of arylcarboxylic acis (8-13) (Table 2)

Reagents: (a) CH_3I in acetone, reflux, (b) several substituted diamines, 60-80 °C; (c) NaH, CuO, 85 °C to 150-160 °C, N_2; (d) MeOH/HCl

Chart 1

Based on the data obtained from docking studies, compounds **2**, **4**, **7**, **8** and **11** had hydrogen bonding ability with amino acid Lys295 of the enzyme active site. Responsible atoms of compounds for H-bonding are shown in Tables 1 and 2. Amino acid residues, which are involved in hydrogen bonding, are placed in Fig. 1. The docking results of enzyme interacted compounds are shown in Table 3.

Among all inhibitors, compound **2** had an interaction with the active site amino acid, Lys295. The binding energy in this interaction was found to be −20.60 kcal/mol. One hydrogen bond was formed within this energy level and this hydrogen bond was belonged to hydrogen of N (2) atom of the compound **2** and N (2) atom of Lys295 with the distance of 2.41 Å (Table 3).

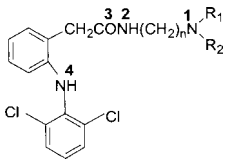
Compound **4** formed two hydrogen bonds against p60$^{c\text{-}Src}$ tyrosine kinase with −10.88 kcal/mol of binding energy. One of the hydrogen bond belonged to Lys295 of the enzyme active site. The first location of hydrogen bond was between oxygen atom of the compound **4** and hydrogen of N (2) atom of Lys295 with the distance of 2.96 Å. The second location of hydrogen bond was between hydrogen of N (4) atom of the compound **4** and N atom of Asp404 with the distance of 2.34 Å (Fig. 2).

The hydrogen bond of compound **7** had the lowest binding energy level with −36.35 kcal/mol energy. Two hydrogen bonds were created between the compound **7** and enzyme active sites. The first bond was between the oxygen atom of the compound **7** and the hydrogen of N (2) atom of Lys295 enzyme with its distance being 2.87 Å and the second bond was between hydrogen of N (4) atom of the compound **7** and the N atom of Asp404 with the distance of 2.13 Å.

Among the carboxylic acid derivatives, compound **8** had hydrogen bond with the active site amino acid, Lys295 with the lowest binding energy of −20.97 kcal/mol. One hydrogen bond was observed between the oxygen atom of compound **8** and the hydrogen of N (2) atom of Lys295 with the distance of 2.97 Å.

Another carboxylic acid derivative **11** also displayed an interaction with Lys295 amino acid by forming three hydrogen

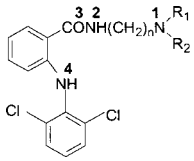
Table 1. Inhibitory Activities of Arylacetic Acid Derivatives Toward Particular Tyrosine Kinase Src (p60$^{c\text{-}Src}$)



Compound	*n*	R_1	R_2	IC_{50} (μM)
1	2	–H	–H	1059
2	2	–H	–Me	814
3	2	–H	–Et	1109
4	2	–Me	–Me	671
5	3	–H	–H	1381
6	3	–Me	–Me	788
7	3	–Et	–Et	925
PP1	—	—	—	170

Superscripts on the chemical formula shows the atoms which can interact with Lys295 and Asp404 amino acids.

Table 2. Inhibitory Activities of Arylcarboxylic Acid Derivatives Toward Particular Tyrosine Kinase Src (p60$^{c\text{-}Src}$)



Compound	*n*	R_1	R_2	IC_{50} (μM)
8	2	–H	–H	983
9	2	–H	–Et	1013
10	2	–H	–i-Pr	4695
11	2	–Me	–Me	909
12	2	–Et	–Et	3508
13	3	–Me	–Me	957
PP1	—	—	—	170

Superscripts on the chemical formula shows the atoms which can interact with Lys295 and Asp404 amino acids.

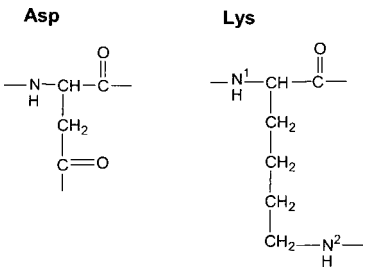


Fig. 1. Amino Acid Residues Involved in Hydrogen Bonding

Superscripts indicate the atom responsible for hydrogen bonding with ligand molecules.

Table 3. Docking Results of Arylacetic Acid and Arylcarboxylic Acid Derivatives

Compound	Energy (kcal/mol)	Against p60$^{c\text{-}Src}$ H-bond (distance Å)					RMSD[a]
2	−20.60	N–H (2)	with	N (2)	Lys295	(2.41)	5.25
4	−10.88	N–H (4)	with	N	Asp404	(2.34)	4.55
		O (3)	with	N–H (2)	Lys295	(2.96)	
7	−36.65	O (3)	with	N–H (2)	Lys295	(2.87)	5.57
		N–H (4)	with	N	Asp404	(2.13)	
8	−20.97	O (3)	with	N–H (2)	Lys295	(2.97)	8.31
11	−14.19	O (3)	with	N–H (2)	Lys295	(2.85)	4.65
		N–H (2)	with	N (2)	Lys295	(2.09)	
		N–H (4)	with	N	Asp404	(2.32)	

a) RMSD: root mean square deviation.

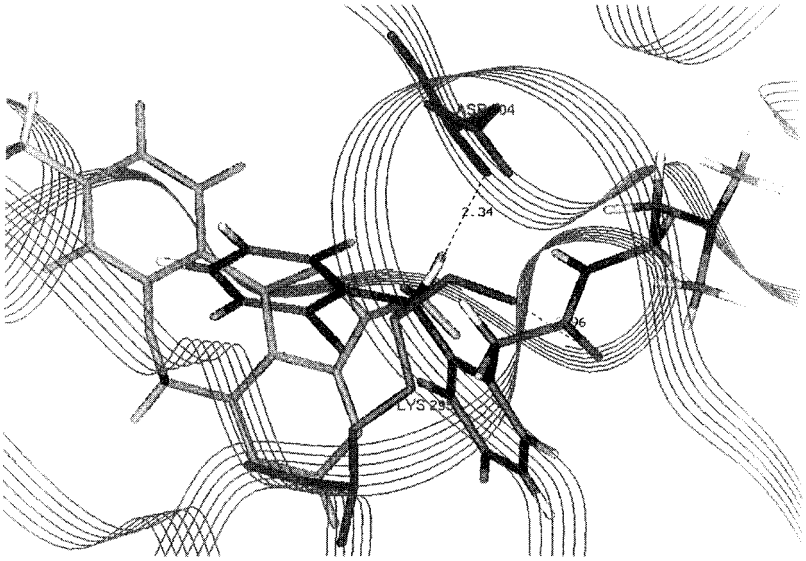


Fig. 2. Docking of the Compound **4** with $p60^{c\text{-}Src}$ Tyrosine Kinase

bonds. The binding energy was found to be −14.19 kcal/mol. The first bond was formed between the oxygen atom of the compound and the hydrogen of N (2) atom of Lys295 with the distance of 2.85 Å. The second hydrogen bond was appeared between the hydrogen of N (2) atom of compound **11** and N (2) atom of Lys295 with the distance of 2.09 Å. The third hydrogen bond was formed between the hydrogen of the N (4) atom of compound **11** and N atom of Asp404 with the distance of 2.32 Å. The RMSD (root mean square deviation) value of compound **4** was found lower (4.55) than the other compounds. Low RMSD value of the compounds place the compounds closer to the active site of the enzyme, the site where the sample inhibitor was manually moved and localized. In other words, since we identified the active site by one of reliable sources, PDBsum (A database of the 3D structures of proteins and nucleic acids, compiled by Roman Laskowski *et al.*, University College London, U.K., http://www.biochem.ucl.ac.uk/bsm/pdbsum/ accessed on December 5, 2002), and an ligand **PP1** (4-amino-5-(4-methylphenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine)[19] was placed manually to that active site, it is considered that the **PP1** is located at the active site of the enzyme ($p60^{c\text{-}Src}$ tyrosine kinase). Therefore, it is generally understood that the lower the RMSD between the **PP1** and a particular inhibitor, the better the binding capacity of the inhibitor. That is, compound **4** had ability to get closer to the active site of the enzyme comparing with the other derivatives and this low RMSD can lead to the increased binding capacity of the compound.

The specific $p60^{c\text{-}Src}$ PTKs inhibitor, **PP1** ($IC_{50}=170\ \mu M$) was used as an effective standard to evaluate the docking mode of compounds against $p60^{c\text{-}Src}$ PTK enzyme active site. All the compounds were shown to be docked at the active site where intact **PP1** was embedded for $p60^{c\text{-}Src}$ PTK.

The arylacetic acid and arylcarboxylic acid derivatives were used for testing the inhibitory activity against tyrosine kinase *in vitro* by ELISA method.[13] According to inhibitory activity results it can be concluded that compound **4** is the most active compound among the entire synthesized compounds tested for the enzyme inhibition.

As far as the docking energy levels are concerned, all compounds in Table 3 showed well low negative values ranging from 10's kcal/mol to 30's kcal/mol. Considering the fact that screening of compounds to check the activity of those compounds is the major task of DOCK 4.0, an important finding is that these energy values are low enough for compounds to be docked into the active site.

RMSD value tells us how far the docked compound is situated from the the ideally docked compound, that is, it shows the superimposability between the two compounds. The lowest value 4.55 was observed in compound **4**, and the highest, in compound **8**. These values are mathematically-calculated value, and the difference in value has its numerical and locational significance.

As far as the hydrogen bond is concerned, all compounds in Table 3 demonstrated the existence of the hydrogen bond with Lys295 which is the active site amino acid in the binding pocket. There is no intramolecular hydrogen bond observed between Lys295 and Asp404. It is important to have the hydrogen bond. The lowest energy does not necessarily give us a stable docking, if the docking energy is more or less scattered evenly so that the docked compound can not stay stable as it should be. On the other hand, if the docked compound demonstrate a hydrogen bond on which docking orientation is created to make the compound stably situated even though the total docking energy is not low as otherwise. Three hydrogen bonds were observed in compound **11**, two in compounds, **4**, **7**, and **8**, and one in compounds **2** and **8**. From this data, compound **4** can be considered one of the strongest inhibitor among the compounds tested.

Taking into consideration all factors involved in the docking simulation observed in the above, compound **4** can be distinguished as the PTK inhibitor from the other tested compounds (Fig. 2), although some unknown variations are yet to be investigated. At the same time the more elaborate docking system and more advanced computer system which will be developed in the near future, hopefully, will produce us a more accurate docking result.

When we examine the structure activity relationship of most effective compounds **4** and **11** with others, it was found

that methyl group is important on the R_1 and R_2 side chain. Besides, another remarkable point of structure activity relationship of arylacetic and arylcarboxylic acid derivatives noted in the $–CH_2$ linkage between aromatic rings and the amide group of side chain which exist only in arylacetic acid derivatives. Nevertheless this group provides more flexibility and bulkiness to the structure and can positively effect the interaction of compounds with the enzyme active site and their activity.

CONCLUSIONS

While increasingly important characteristics of protein tyrosine kinase, especially a src family in such diseases as cancer and AIDS, there still a quite big challenge remains to develop or find a site specific inhibitors of those enzymes. In this paper, was investigated inhibitory activity of previously synthesized arylacetic and arylcarboxylic acid derivatives against $p60^{c\text{-}Src}$ tyrosine kinase by the ELISA method *in vitro*. At the same time, docking mode between the inhibitors and the enzyme was examined. It was found that the inhibitory activity of compounds became higher when the $–CH_2$ linkage exists between aromatic ring and the amide group of the side chain. Furthermore, the higher inhibitory activity was noted for the compounds with the R_1 and R_2 substitutents being methyl group in both series, The result of the docking study by the use of DOCK 4.0 showed that compounds **2**, **4**, **7**, **8**, **11** render satisfactory interaction with the active site of enzyme, Lys295 of $p60^{c\text{-}Src}$ tyrosine kinase. Observation of the docking interaction and the evaluation of biological data indicated that compound **4** is the most active among the entire derivatives.

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