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Evaluation of Indole Esters As Inhibitors of p60$^{c\text{-}Src}$ Receptor Tyrosine Kinase and Investigation of the Inhibition Using Receptor Docking Studies

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Several indole esters were tested as inhibitors of tyrosine kinase p60$^{c\text{-}Src}$. Compound (4) was found fairly active against the enzyme with $IC_{50} = 1.34\,\mu M$. DOCK methodology was used to asses our inhibitors for their inhibitory potency against tyrosine kinase. The docking results showed that compounds (4), (25) and (26) were bound to the active site of the enzyme Lys 295 of p60$^{c\text{-}Src}$ tyrosine kinase. Both activity and docking studies showed a parallel result, with compound (4) having a better interaction with the enzyme active site and also greater activity than the other compounds, indicating a potential role as new lead inhibitor.

Keywords: Tyrosine kinase; Inhibition; Docking; Indole esters; Active site; Enzyme interaction; p60$^{c\text{-}Src}$

INTRODUCTION

Tyrosine kinase inhibitors (TKIs) are an important group of compound for the development of small molecule therapies against angiogenesis.[1] Since TKIs have the capability to directly block growth factor signaling in the course of angiogenesis,[2] targeting TKIs could lead to inhibition of tumor growth.[3] Some indole derivatives have been found as inhibitors for a number of RTKs and are thought to be involved in angiogenesis, particularly the VEGF receptors.[4–6] Among indole derivatives, **SU5416** (Figure 1) was developed by the SUGEN company and is currently in clinical trials.[7,8]

Since some indole derivatives were found to be highly effective against PTKs, we aimed to evaluate some previously reported indole esters[9] (Table 1) as inhibitors against p60$^{c\text{-}Src}$ PTK enzyme. The p60$^{c\text{-}Src}$ belongs to the non-receptor-linked membrane-anchored PTKs (scr-family PTKs). This enzyme has been implicated in the development of leukemia, breast, and colon cancer.[10] The p60$^{c\text{-}Src}$ PTK inhibitor **PP1** ($IC_{50} = 170\,nM$) was used as an effective standard compound to evaluate the activity of our compounds against p60$^{c\text{-}Src}$ PTK.

Molecular docking techniques that can help to predict lead compounds are frequently used the in the development of medicinal drugs. In this study, we used the DOCK 4.0 program to evaluate the interactions of our indole derivatives with the active site of the p60$^{c\text{-}Src}$ enzyme.

MATERIALS AND METHODS

Docking Study

The DOCK 4.0 program,[11] was utilized for the identification of specific binding inhibition properties of our indole derivatives on tyrosine kinase enzyme p60$^{c\text{-}Src}$. The docking experiments as well as receptor and ligand preparations were performed on a SGI Indigo Extreme (R4400) workstation. Insight II software (MSI)[12] was used for drawing compounds. To attach hydrogen atoms,

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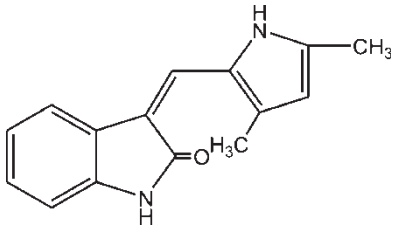


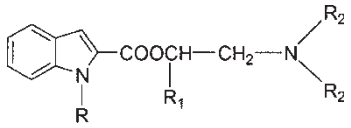
FIGURE 1 The indole derivative **SU 5416**; a TKIs inhibitor.

molecules were converted to the Sybyl (Tripos) mol2 files. PP1 was used as the ligand and was 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, 2ptk ($p60^{c\text{-}Src}$) was taken from PDB (Protein Data Bank) and placed into the Insight II where docking with inhibitors were conducted. The tyrosine kinase $p60^{c\text{-}Src}$ used for docking was chicken SRC tyrosine kinase, and the magnitude of resolution was 2.35 Å. The three dimensional structures were obtained by X-ray diffraction analysis.

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.[12] 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 rotation of ligand atoms. 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.

The amino acid residues representing the active site of tyrosine kinase $p60^{c\text{-}Src}$, where the pocket is created, were considered at Lys 295 and Glu 310.[13] As far as the geometry of the protein is concerned,

TABLE I Structural formulas of compounds (**1**–**27**) and inhibitory potency against $p60^{c\text{-}Src}$ tyrosine kinase



Compd	Type of salt	R	R_1	R_2	IC_{50}, μM*
1	HCl	CH_2Ph	H	CH_3	>500
2	HCl	CH_2Ph	H	C_2H_5	>1000
3	HCl	CH_2Ph	H	Pyrrole	>1000
4	HCl	CH_2Ph	H	Piperidine	1.34
5	HCl	CH_2Ph	CH_3	CH_3	>1000
6	CH_3I	CH_2Ph	H	CH_3	630.97
7	CH_3I	CH_2Ph	H	C_2H_5	614.00
8	CH_3I	CH_2Ph	H	Pyrrole	215.01
9	CH_3I	CH_2Ph	H	Piperidine	512.56
10	CH_3I	CH_2Ph	CH_3	CH_3	>1000
11	HCl	Ph	H	CH_3	>1000
12	HCl	Ph	H	C_2H_5	>1000
13	HCl	Ph	H	Pyrrole	258.42
14	HCl	Ph	H	Piperidine	306.18
15	HCl	Ph	CH_3	CH_3	>1000
16	HCl	Ph	H	Piperazine	>1000
17	CH_3I	Ph	H	CH_3	>1000
18	CH_3I	Ph	H	C_2H_5	816.66
19	CH_3I	Ph	H	Pyrrole	>1000
20	CH_3I	Ph	H	Piperidine	278.92
21	CH_3I	Ph	CH_3	CH_3	>1000
22	HCl	H	H	C_2H_5	>1000
23	CH_3I	H	H	C_2H_5	>1000
24	HCl	H	H	Pyrrole	21.43
25	HCl	H	H	Piperidine	829.63
26	HCl	H	CH_3	CH_3	>500
27	HCl	H	H	Piperazine	>500
PP1					0.17

* Values were obtained from at least three independent experiments.

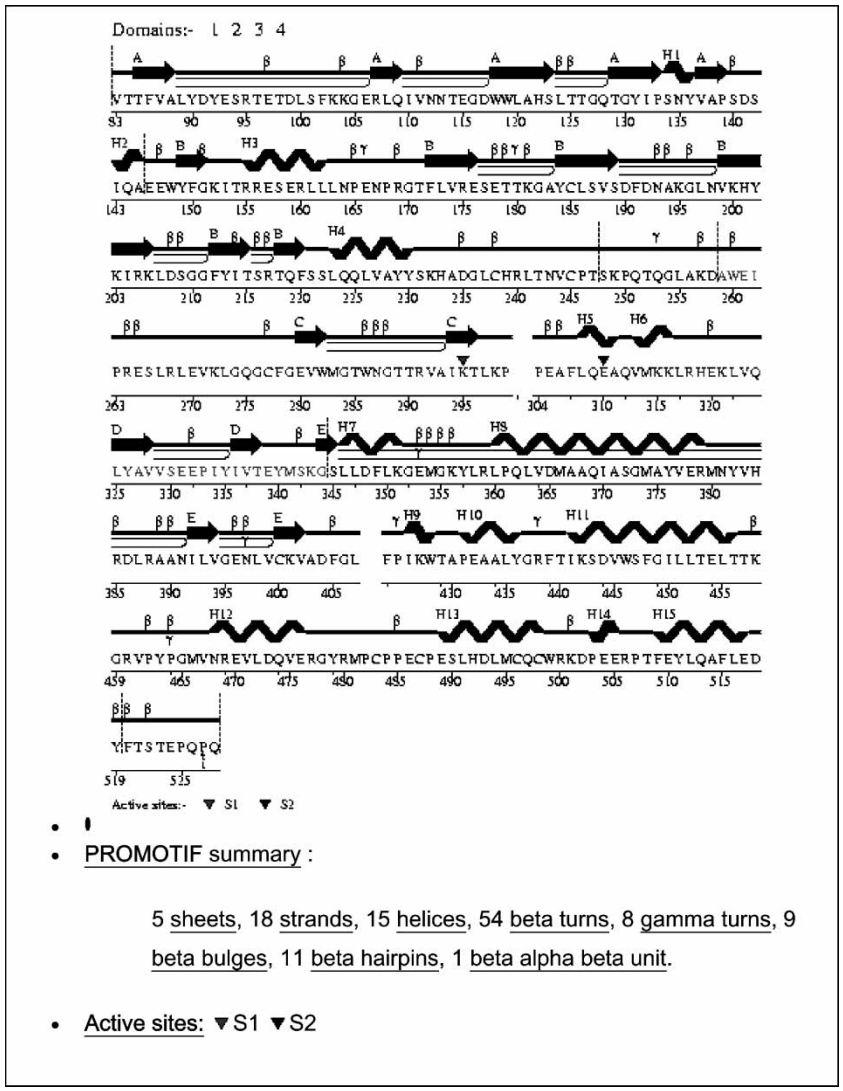


FIGURE 2 Secondary structure[13] representation of chicken SRC tyrosine kinase p60$^{c\text{-}Src}$.

it consists of 5 sheets, 15 helices, 54 β turns, 8 γ turns, and 11 hairpins (Figure 2). Based on the information on the pocket, the inhibitor was placed in the pocket manually. DOCK 4.0, filled the pocket with spheres, moved the 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.

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

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

Tyrosine Kinase Assay

Assay Kit

Takara Universal Tyrosine Assay Kit (Takara-bio co.) was used to test the synthesized compounds. This assay kit determines the inhibition activities of inhibitors against a substrate of tyrosine kinase.[14]

The contents of the kit consists of PTK substrate immobilized microplate (8 well × 12), kinase reacting

solution (11.0 mL), 40 mM ATP-2Na (0.55 mL), extraction buffer (11.0 mL), PTK control (0.50 mL), anti phosphotyrosine (PY20-HRP, for 5.5 mL/H_2O), blocking solution (11.0 mL), HRP coloring solution (TMBZ = tetra methyl benzidine, 12.0 mL).

Other reagents and materials used were: *N*,*N*-Dimethylformamide (sample solvent, Wako Pure Chemical Industries Ltd.), 1 N sulfuric acid (to stop chlorination reaction, Wako Pure Chemical Industries Ltd.), 0.05% tween in PBS (phosphate-buffered saline, Wako Pure Chemical Industries Ltd.), 2-mercaptoethanol (ICN Biomedicals Inc.), 37°C incubator (Masuda SA-30), micro TAITA plate reader (λ450 nm neighboring filter, BioRad 550).

Immunoassay

40 μL of tyrosine kinase and inhibitors were placed into the wells of a 96-well microtiter plate, respectively. To starting the phosphorylation reaction, 10 μL of 40 mM ATP was added into each well and then the plate was incubated at 37°C for 30 min. After reaction, the liquid was disposed of and the plate was washed (×4) with Tween-PBS. The plate was turned over on a paper towel to remove the remaining solution after each washing. A blocking solution (100 μL) was added to the wells and the plate was incubated at 37°C for 30 min. The solvent was decanted and turned over on the paper towel to remove the remaining solvent. Anti-phosphotyrosine (50 μL) was added to the vessels and the plate was incubated at 37°C for 30 min. The reaction liquid was removed and the plate washed (×4) with Tween-PBS. The remaining liquid was thoroughly decanted on a paper towel each time. HRP coloring agent (100 μL) was added into every well, incubated at 37°C for 15 min, and the reaction stopped by the addition of 100 μL/well 1 N sulfuric acid and the absorbance measured at λ450 nm in a microplate reader.

RESULTS

Through docking analysis, we have identified the interaction of our active inhibitors with the enzyme. Based on the obtained data, the docking results for the indole esters were evaluated and are shown in Table 2. Atoms responsible for H-bonding of compounds are shown in Figure 3.

Computational docking results predicted that compound (**4**) has good interaction with the enzyme active site compared to the others. Compound (**4**) formed two hydrogen bonds

TABLE II Docking result of indole derivatives

Indole derivatives	Energy (kcal/mol)	Against c-Src p60 H-bond (distance A°)	RMSD (root mean square deviation)
1	01. − 11.35	17. N-2 with O-2 of Met 314 (2.98) N-2 with O-2 of Leu 317 (2.70)	5.36
	02. − 5.77	18. N-2 with O-2 of Tyr 340 (1.52) O-2 with N-3 of Met 341 (2.96)	11.82
	03. − 3.82	19. O-2 with N-3 of Met 341 (2.96) N-2 with O-2 of Met 341 (1.65) N-2 with O-3 of Ser 342 (3.00)	12.29
4	01. − 13.77	15. O-2 with O-1 of Thr 338 (2.93)	23.60
	02. − 9.81	16. O-1 with N-4 of Lys 295 (2.90) O-1 with O-5 of Asp 404 (2.98)	22.90
	03. − 9.75	17. O-1 with N-4 of Lys 295 (2.90) O-1 with O-5 of Asp 404 (2.98)	22.92
13	01. − 17.84	22. N-2 with O-2 of Val 323 (2.59)	5.81
	02. − 17.75	23. N-2 with O-2 of Met 314 (2.72)	7.04
14	01. − 15.90	18. O-1 with O-5 of Asp 404 (2.62)	8.35
	02. − 13.31	21. N-2 with O-2 of Met 314 (2.51)	5.84
	03. − 12.61	22. N-2 with O-2 of Leu 317 (2.34)	5.03
24	01. − 26.35	14. O-1 with O-1 of Thr 338 (2.96)	6.76
	02. − 26.13	15. O-1 with O-1 of Thr 338 (2.96)	6.79
	03. − 25.86	19. O-2 with O-1 of Thr 338 (2.97)	7.72
25	01. − 27.13	01. O-1 with N-4 of Lys 295 (2.97)	7.71
	02. − 26.83	02. O-1 with N-4 of Lys 295 (2.97)	7.70
	03. − 25.54	10. O-1 with N-4 of Lys 295 (2.97)	7.87
	04. − 14.69	20. N-1 with O-5 of Asp 404 (2.44)	7.58
	05. − 14.15	21. O-1 with O-1 of Thr 338 (2.79)	8.81
26	01. − 19.38	18. O-1 with N-4 of Lys 295 (2.99)	7.70
	02. − 3.72	23. N-2 with O-1 of Thr 338 (2.78)	7.31

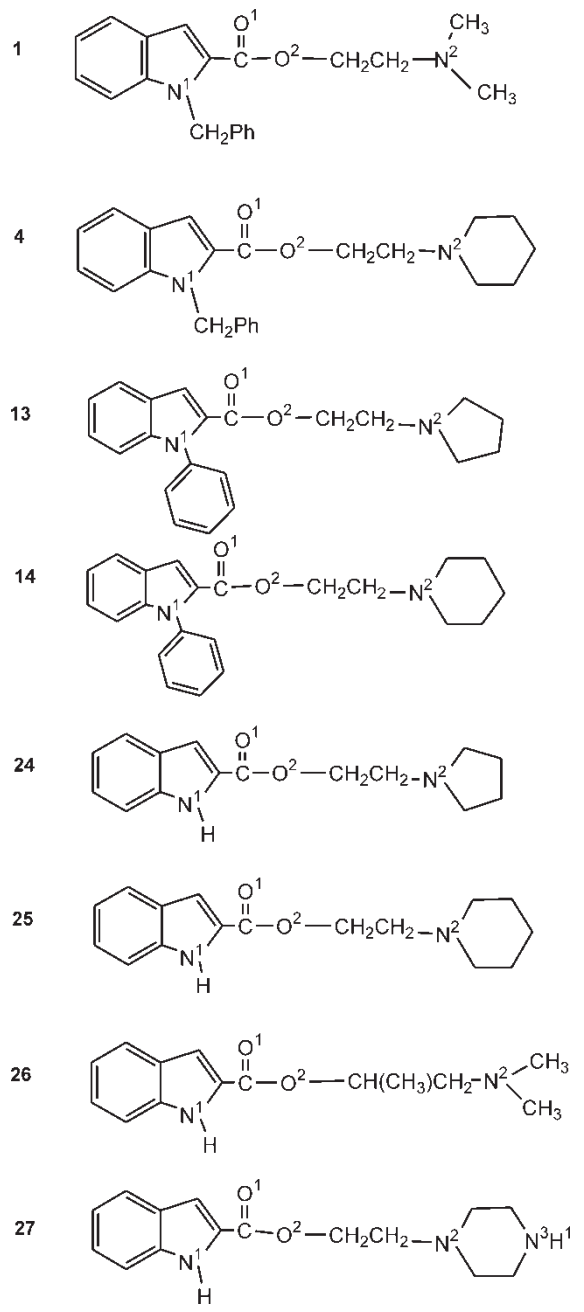


FIGURE 3 Indole derivatives studied for docking mode and atoms responsible for H-bonding with 2ptk (p60$^{c\text{-}Src}$).

with the p60$^{c\text{-}Src}$ tyrosine kinase, while its binding energy was −9.81 kcal/mol. One of the hydrogen bonds belongs to the active site Lys 295. This first located hydrogen bond was formed between the oxygen (C═*O*) of compound (**4**) and the nitrogen (NH_2) of Lys 295 with a distance of 2.90 Å (Figure 4). The second located hydrogen bond was formed between the oxygen (C═*O*) of compound (**4**) and the hydrogen (O–*H*) of Asp 404 with a distance of 2.98 Å. It was also found that the other active compound (**24**) has a H bond which is formed between the oxygen (C═*O*) of compound and hydrogen (O–*H*) of Thr 338 with its distance of 2.96 Å.

Among the indole derivatives compound (**4**) showed the strongest inhibition with an IC_{50} value of 1.34 μM. The docking result for this compound revealed that a hydrogen bond existed between Lys 295 and carbonyl oxygen of compound (**4**) in the 16th lowest docking ranking where docking energy was −9.81 kcal/mol (Table 2). Another hydrogen bond with the carbonyl oxygen was observed with Asp 404 in the same docking mode of the 16th lowest docking ranking with an energy of −9.81 kcal/mol, which is sufficiently low as an inhibitory energy. In the 17th lowest docking ranking, the similar docking mode observed also created a hydrogen bond, and the docking energy was −9.75 kcal/mol. No other compounds exhibited two hydrogen bonds with one of them being with the active site, Lys 295. This explains the fact that compound (**4**) showed the strongest inhibition with an IC_{50} of 1.34 μM.

Compounds were evaluated for their inhibitory activity toward tyrosine phosphorylation activities associated with p60$^{c\text{-}Src}$ tyrosine kinase (Table 1). IC_{50} values were defined as the concentration of a compound required to achieve 50% inhibition of tyrosine kinase activity compared to vehicle-treated controls (DMSO). Compounds with IC_{50} values >50 μM were considered inactive.

Concerning the structure–activity-relationship between indole derivatives and p60$^{c\text{-}Src}$ tyrosine kinase, the methyl substitutions at R_2 gave less inhibitory potency than the other more bulky substituents such as pyrrole and piperidine. Methyl substitution at R_1 position gave inactive compounds in all cases suggesting the existence of steric hindrance around the R_2 region. In terms of the type of salts, the iodide did not have a noticeable potency compared with the hydrochloride.

Compound (**4**) represented the most potent inhibitor of p60$^{c\text{-}Src}$ tyrosine kinase in this study (IC_{50} = 1.34 μM, Table 1). In addition compound (**24**) also had good inhibitory potency against tyrosine kinase with an IC_{50} value of 21.43 μM. However, compounds (**8**), (**13**) and (**20**) were found 200-fold less potent than compound (**4**).

Computational studies suggested that the good inhibitory potency of compound (**4**) might be the result of a favorable interaction with the active site Lys 295. Both activity and docking studies showed a parallel result in that compound (**4**) has a better interaction with the enzyme active site and it is also a more potent inhibitor than the other compounds studied.

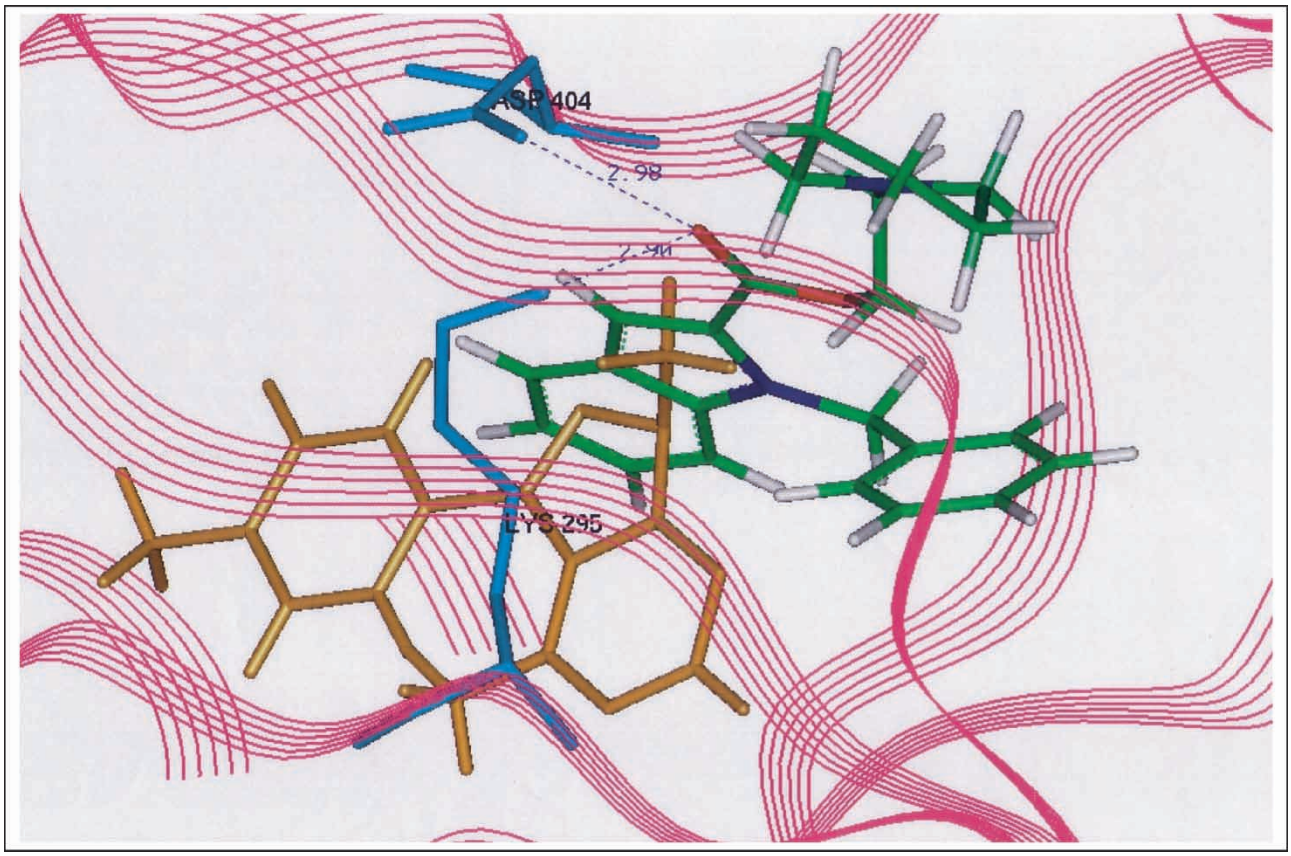


FIGURE 4 Interactions of compound **4** (green) and PP1 (orange) with tyrosine Kinase $p60^{c\text{-}Src}$.

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