4-Aryl-4*H*-Chromene-3-Carbonitrile Derivatives: Evaluation of Src Kinase Inhibitory and Anticancer Activities

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Abstract: Src kinase mutations and/or overexpression have been implicated in the development of a number of human cancers including colon, breast, and lung cancers. Thus, designing potent and selective Src kinase inhibitors as anticancer agents is a subject of major interest. A series of 4-aryl substituted derivatives of 2-amino-7-dimethylamino-4*H*-chromene-3-carbonitrile were synthesized using one-pot reaction of appropriate substituted aromatic aldehydes, malononitrile, and 3-(dimethylamino)phenol in the presence of piperidine. All 23 compounds were evaluated for inhibition of Src kinase and cell proliferation in human colon adenocarcinoma (HT-29) and leukemia (CCRF-CEM) cell lines. Among the tested compounds, 2-chlorophenyl- (**4c**), 3-nitrophenyl- (**4h**), 4-trifluoromethyphenyl- (**4i**), and 2,3-dichlorophenyl- (**4k**) substituted chromenes showed Src kinase inhibitory effect with IC_{50} values of 11.1-18.3 μM. Compound **4c** was relatively selective against Src (IC_{50} = 11.1 μM), when compared with selected kinases, epidermal growth factor receptor (EGFR, IC_{50} > 300 μM), C-terminal Src kinase (Csk, IC_{50} = 101.7 μM), and lymphocyte-specific protein tyrosine kinase (Lck, IC_{50} = 46.8 μM). 3-Chlorophenyl substituted thiazole (**4v**) and 2-chlorophenylsubstituted thiazole (**4u**) chromene derivatives inhibited the cell proliferation of HT-29 and CCRF-CEM by 80% and 50%, respectively, at a concentration of 50 μM. The data indicate that 4*H*-chromene-3-carbonitrile scaffold has the potential to be optimized further for designing more potent Src kinase inhibitors and/or anticancer lead compounds.

Keywords: Anticancer activity, benzopyranones, carbonitrile, chromenes, inhibitor, protein kinase, Src kinase.

INTRODUCTION

Protein tyrosine kinases (PTKs) catalyze the transfer of γ-phosphate group from ATP to specific tyrosine residue of many protein substrates. Src family tyrosine kinases (SFKs) are subject of major interest because of their critical roles in signal transduction pathways and cellular functions, such as division, differentiation, survival, adhesion, migration, and invasion. SFKs are a family of eleven different non-receptor PTKs, including Blk, Brk, c-Fgr, Frk, Fyn, Hck, Lck, Lyn, c-Src (Src), Srm, c-Yes [1] of which Src is the prototype. There is significant evidence demonstrating that overexpression or mutation of Src kinase correlate with tumor growth and metastasis in several human malignancies, including colon, breast and pancreatic cancers [2-6]. Src has also been implicated to have key roles in other pathologic disorders, such as osteoporosis, cardiovascular diseases, and neurodegeneration [7]. Thus, Src kinase has become an attractive target for designing new lead compounds against cancer and other diseases.

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Over the years, a diverse number of compounds have been identified as Src kinase inhibitors [8]; among them there are several different structural classes, including staurosporine as a standard nonselective protein kinase inhibitor, pyrazolopyrimidine (e.g., PP1, PP2) [9,10], anilinoquinazoline [11,12], and quinolinecarbonitrile [13-15] derivatives. The Saracatinib (AZD0530) [12,16] and Bosutinib (SKI-606) [17,18] with anilinoquinazoline and 3-quinolinecarbonitrile cores, respectively (Fig. **1**), are among potent Src kinase inhibitors, which are currently in clinical development for the treatment of a wide range of tumor types. Designing selective Src kinase inhibitors still remains a challenge. Because of the highly conserved nature of the ATP-binding site within the Src family kinase, nonselective inhibitors may provoke toxicity *in vivo*. Thus, additional research is needed to identify new chemical scaffolds that can inhibit selectively Src kinase domain.

There is evidence that the presence of a nitrile group in different series of compounds like quinolinecarbonitrile (e.g., Bosutinib), and benzylidene-malononitriles is crucial for protein kinase inhibitory activity [19]. Within this category, compound A (Fig. **2**) [20] has been reported as an inhibitor of Lck (IC_{50} = 7-10 μM). Dicycanosubstituted compound B (Fig. **2**) [21] has been shown to reduce the steady-state levels of Src in NIH 3T3 cells.

Furthermore, several natural benzopyranone-containing compounds (flavonoids), such as quercetin and genistein

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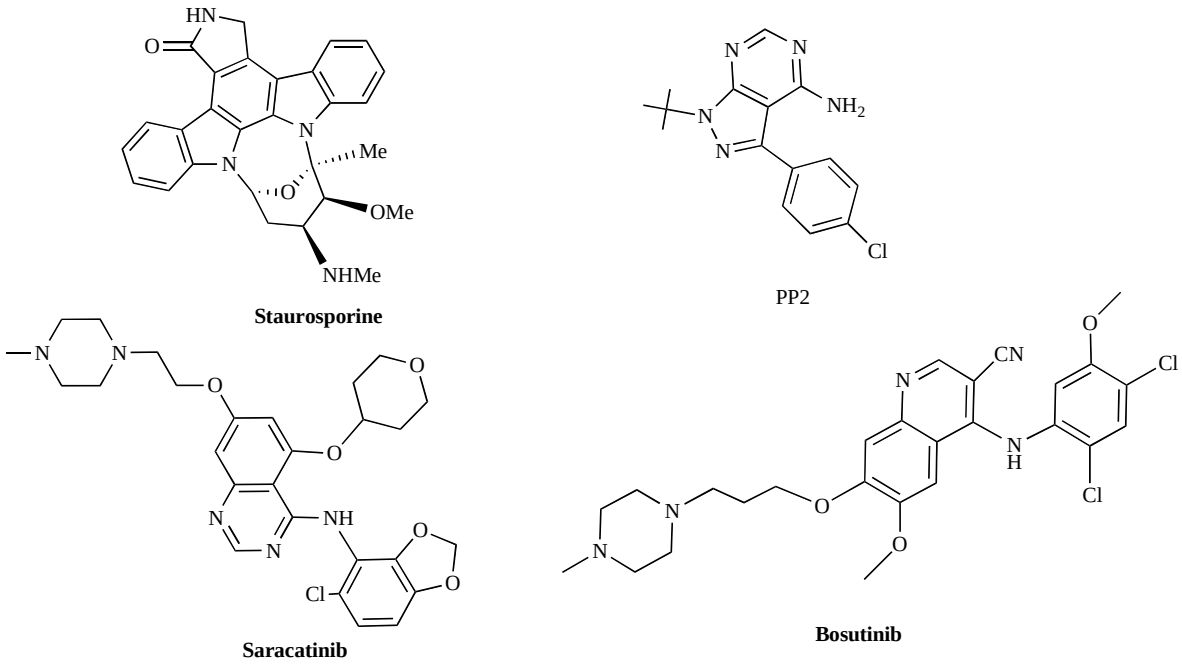


Fig. (1). Chemical structures of some of the Src kinase inhibitors.

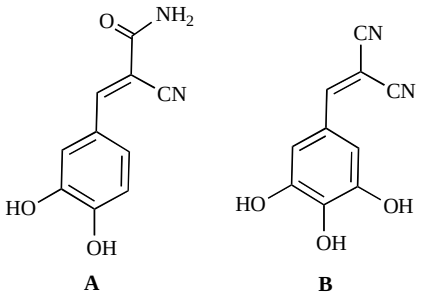


Fig. (2). Benzylidene-malononitrile derivatives as PTK inhibitors.

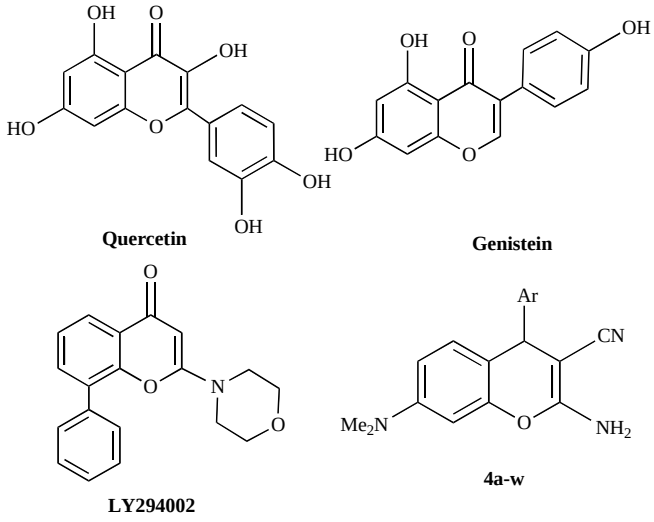


Fig. (3). Chemical structures of natural protein kinase inhibitors containing benzopyranone core (quercetin and genistein), chormene core (LY294002), and synthesized 4-aryl-4*H*-chromene-3-carbonitrile derivatives (**4a-w**).

(Fig. **3**) [19] have been shown to serve as general inhibitors for protein kinases. Using quercetin as a model compound, several chromenes such as LY294002 (Fig. **3**) [22] were synthesized and evaluated for their ability to inhibit tyrosine kinases. LY294002 was found to be a potent and selective inhibitor of PtdIns 3-kinase.

Considering the fact that a series of variously substituted 4-aryl-4*H*-chromens-3-carbonitrile have anticancer and apoptosis-inducing effect [23,24] and in continuation of our efforts to design or identify new scaffolds as kinase inhibitors [25-32], herein we report the synthesis and evaluation of selected 2-amino-4-aryl-7-dimethylamino-4*H*-chromene-3-carbonitrile derivatives **4a-w** (Fig. **3**) that contain both nitrile and chromene core for their protein kinase inhibitory activity.

MATERIALS AND METHODS

1. General

All starting materials, reagents, and solvents were purchased from Merck AG (Germany). The purity of the synthesized compounds was confirmed by thin layer chromatography (TLC) using various solvents of different polarities. Merck silica gel 60 F_{254} plates were applied for analytical TLC. Column chromatography was performed on Merck silica gel (70–230 mesh) for purification of the intermediate and final compounds. Melting points were determined on a Kofler hot stage apparatus (Vienna, Austria) and are uncorrected. ^{1}H NMR spectra were recorded using a Bruker 500 MHz spectrometer (Bruker, Rheinstatten, Germany), and chemical shifts are expressed as d (ppm) with tetramethylsilane (TMS) as an internal standard. The IR spectra were obtained on a Shimadzu 470 (Shimadzu, Tokyo, Japan) spectrophotometer (potassium bromide disks). The mass spectra were run on a Finnigan TSQ-70 spectrometer (Thermo Finnigan, West Chester, OH, USA) at 70 eV. Elemental analyses were carried out on a CHN-O-rapid elemental analyzer (Heraeus GmbH, Hanau, Germany) for C, H, and N, and the results were within ± 0.4% of the theoretical values.

2. General Procedure for the Preparation of 2-amino-4-aryl-7-dimethylamino-4*H*-chromene-3-carbonitrile derivatives 4a-w

In general, piperidine (10 mmol) was added to a mixture of the 3-dimethylaminophenol (5 mmol), substituted aryl-benzaldehyde (5 mmol), and malonitrile (5 mmol) in ethanol (20 mL). The reaction mixture was stirred at 35 ºC for 12 h. After cooling, the precipitated solid was filtered, washed with cold ethanol, and crystallized from the same solvent. The synthesized compounds were characterized by IR, ^{1}H NMR, mass spectral data and elemental analysis. Physico-chemical and spectral properties of compounds **4a-w** were consistent with the previously reported data [23,24,33].

3. c-Src Kinase Activity Assay

The effect of synthesized compounds on the activity of c-Src kinase was determined by HTScan Src Kinase Assay Kit, catalogue number 7776 from Cell Signaling Technology (Danvers, MA, USA); according to manufacturer's protocol. Streptavidin-coated plates were purchased from Pierce (Rockford, IL, USA). In brief, the kinase reaction was started with the incubation of the 12.5 µL of the reaction cocktail (0.5 ng/µL of GST-Src kinase in 1.25 mM DTT) with 12.5 µL of prediluted compounds (dissolved in 1% DMSO) for 5 min at room temperature. ATP/substrate (25 µL, 20 µM/1.5uM) cocktail was added to the mixture. The biotinylated substrate (catalogue number 1366) contains the residues surrounding tyrosine 160 (Tyr160) of signal transduction protein and has a sequence of EGIYDVP. The reaction mixture was incubated for 30 min at room temperature. The kinase reaction was stopped with the addition of 50 µL of 50 mM EDTA (pH 8.0). The reaction solution (25 µL) was transferred into 96-well streptavidin plates (Pierce, part number 15125), diluted with 75 µL double distilled water, and incubated at room temperature for 60 min. At the end of the incubation, the wells were washed three times with 200 µL of 0.05% Tween-20 in PBS buffer (PBS/T). After that to the each well was added 100 µL of phosphotyrosine antibody (P-Tyr-100) (1:1000 dilution in PBS/T with 1% BSA) and the wells were incubated for another 60 min. After washing three times with 0.05% Tween-20 in PBS/T, the wells were incubated with 100 µL secondary anti-mouse IgG antibody, which was HRP-conjugated (1:500 dilution in PBS/T with 1% BSA) for next 30 min at room temperature. The wells were washed five times with 0.05% Tween-20 in PBS and then were incubated with 100 µL of 3,3',5,5'-tetramethylbenzidine dihydrochloride (TMB) substrate for 5 min. The reaction was stopped by adding 100 µL/well of stop solution to each well and mixed well and read the absorbance at 450 nm using a microplate reader (Molecular devices, spectra Max M2). IC_{50} values of the compounds were calculated using ORIGIN 6.0 (origin lab) software. IC_{50} is the concentration of the compound that inhibited enzyme activity by 50%. All the experiments were carried out in triplicate.

4. Selectivity Assay of 4c against Csk, EGFR, and Lck

The inhibitory activity of compound **4c** was determined against Csk, EGFR, and Lck according to the KinaseProfiler protocol from Millipore described in www.millipore.com/drugdiscovery/KinaseProfiler. In brief, Csk (h) was incubated with 50 mM Tris (pH 7.5), 0.1 mM EDTA, 0.1 mM Na_3VO_4, and 0.1% β-mercaptoethanol. EGFR (h) and activated Lck (h) were incubated with 8 mM MOPS (pH 7.0) and 0.2 mM EDTA. To the mixture was added substrate (0.1 mg/mL poly(Glu, Tyr) 4:1 for Csk and EGFR, 500 µM KVEKI-GEGTYGVVYK for Lck), 10 mM MgAcetate, and [γ-^{33}P-ATP] (specific activity approx. 500 cpm/pmol, 10 µM). The reactions were initiated by the addition of the MgATP mix. After incubation for 40 min at room temperature, the reaction is stopped by the addition of 3% phosphoric acid solution. 10 µL of the reaction is then spotted onto a P30 filtermat and washed three times for 5 minutes in 75 mM phosphoric acid and once in methanol prior to drying and scintillation counting.

5. CELL CULTURE AND CELL PROLIFERATION ASSAY

5.1. Cell Culture

Human colon adenocarcinoma HT-29 (ATCC no. HTB-38) and leukemia CCRF-CEM (ATCC no. CCL-119) cell lines were obtained from American Type Culture Collection. Cells were grown on 75 cm^2 cell culture flasks with RPMI-16 medium for leukemia and EMEM medium for colon adenocarcinoma and supplemented with 10% fetal bovine serum (FBS), and 1% penicillin-streptomycin solution (10,000 units of penicillin and 10 mg of streptomycin in 0.9 % NaCl) in a humidified atmosphere of 5% CO_2, 95% air at 37 ºC.

5.2 Cell Proliferation Assay

Cell proliferation assay of compounds was evaluated in HT-29 and CCRF-CEM cells, and was compared with that of doxorubicin (DOX). Cell proliferation assay was carried out using CellTiter 96 aqueous one solution cell proliferation assay kit (Promega, USA). Briefly, upon reaching about 75-80% confluency, 5000 cells/well were plated in 96-well microplate in 100 µL media. After seeding for 72 h, the cells were treated with 50 µM compound in triplicate. DOX (10 µM) was used as the positive control. Incubation was carried out at 37 ºC in an incubator supplied with 5% CO_2 for 72 h. At the end of the sample exposure period (72 h), 20 µL CellTiter 96 aqueous solution was added. The plate was returned to the incubator for 1 h in a humidified atmosphere at 37 ℃. The absorbance of the formazan product was measured at 490 nm using microplate reader. The blank control was recorded by measuring the absorbance at 490 nm with wells containing medium mixed with CellTiter 96 aqueous solution but no cells. Results were expressed as the percentage of the control (DMSO without compound set at 100%).

6. Molecular Modeling

Simulations were performed with the Accelrys Discovery Studio 2.5 modeling package, with the CHARMm-based force field [34]. Model of PP1 bound to Src was constructed based on the X-ray crystal structures of PP1 bound to Hck (1QCF) and AMP-PNP bound to c-Src (2SRC) templates from RCSB Protein Data Bank. The coordinates and positions of the backbone atoms of PP1 was superimposed on the corresponding atoms in AMP-PNP after which Hck was deleted. For refinement, the PP1-Src complex underwent

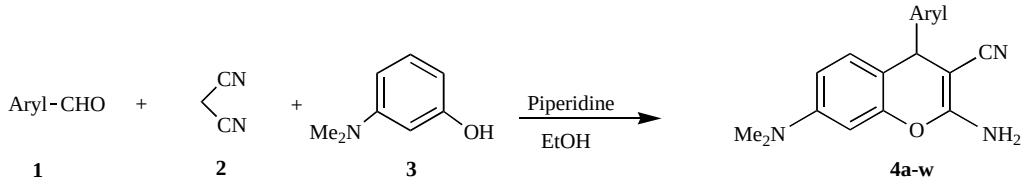


Fig. (4). One-pot synthesis of 4-aryl-4*H*-chromenes **4a-w**.

CHARMm minimization. All default parameters were used in the minimization process.The complex first underwent 50 steps of steepest descent and 150 steps of conjugate gradient minimization. Next, the complex was equilibrated briefly with 200 steps molecular dynamics each at 300 K. The final structure was then minimized with 300 steps with the conjugate gradient algorithm. For the molecular modeling of **4c**, after initial minimization, the coordinates and positions of the backbone atoms of 4-(2-chlorophenyl) group was superimposed on the corresponding atoms in PP1 in complex with c-Src after which PP1 was deleted. The minimization was carried out as described above.

RESULTS AND DISCUSSION

Chemistry

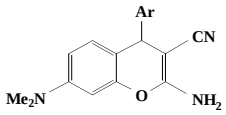
An array of 23 diversely 4-aryl substituted derivatives of 2-amino-7-dimethylamino-4*H*-chromene-3-carbonitrile were synthesized and evaluated against Src kinase. The compounds were synthesized according to the previously reported general procedure by a one-pot reaction [23,24,33] through the condensation of a substituted aromatic aldehyde (**1**), malonitrile (**2**), and 3-(dimethylamino) phenol (**3**) in ethanol in the presence of piperidine (Fig. **4**).

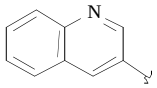
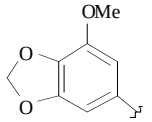
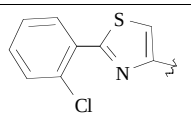
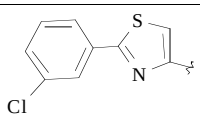
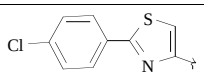
Src Kinase Inhibitory Activity

The results of Src kinase inhibitory activity of compounds **4a-w** are shown in Table **1**. Compounds **4c**, **4h**, **4i**, and **4k** exhibited higher Src inhibitory activity (IC_{50} =11.1–18.3 μM) when compared with other compounds. 2-Chlorophenyl substituted analog **4c** showed an IC_{50} value of 11.1 μM and was the most potent compound in this series. In comparison, 3-chlorophenyl substituted analog **4d** demonstrated 3-fold decrease in inhibitory potency when compared to **4c**. It is noteworthy that movement of chlorine atom to position 4 of phenyl ring in compound **4e** led to loss of activity (IC_{50} >150 μM). Similarly 4-phenylsubstituted analogs **4b** (Ar = 4-F- Ph), **4j** (Ar = 4-CF_3-Ph), and **4p** (Ar = 2,3,4-tri(MeO)-Ph) showed less inhibitory potency when compared with the corresponding 2- or 3-phenylsubstituted analogs **4a** (Ar = 3-F-Ph), **4i** (Ar = 3-CF_3-Ph), and **4n** (Ar = 2,3-di(MeO)-Ph), respectively. Other analogs with chlorophenylthiazolyl bulky groups (**4u-w**) did not show any significant inhibitory activity (IC_{50} > 300 μM) against Src. These data suggest that incorporation of bulky phenylsubstituted thiazole groups or 4-aryl groups with substitution at position 4 is less tolerated in the ATP-binding site cavity of the Src kinase domain.

2-Chloro- (**4c)**, 2,3-dichloro- (**4k**), and 2,6-dichloro- (**4l)** substituted analogs with IC_{50} values of 11.1-23.9 μM containing an electron withdrawing group showed significantly

Table 1. Inhibition of Src Kinase Activity by 2-amino-7-dimethylamino-4*H*-chromene-3-carbonitrile Derivatives



Compounds	Ar	IC_{50} (μM)[a]
4a	3-F-Ph	21.1
4b	4-F-Ph	37.1
4c	2-Cl-Ph	11.1
4d	3-Cl-Ph	32.3
4e	4-Cl-Ph	>150
4f	3-Br-Ph	37.0
4g	2-NO_2-Ph	31.1
4h	3-NO_2-Ph	18.3
4i	3-CF_3-Ph	13.0
4j	4-CF_3-Ph	26.1
4k	2,3-di(Cl)-Ph	18.2
4l	2,6-di(Cl)-Ph,	23.9
4m	2-MeO-Ph	44.6
4n	2,3-di(MeO)-Ph	45.7
4o	2,5-di(MeO)-Ph	>150
4p	2,3,4-tri(MeO)-Ph	>300
4q	2,4-diMe-Ph	>150
4r	4-bi-Ph	28.8
4s		32.7
4t		30.4
4u		>300
4v		>300
4w		>300
Staurosporine	-	0.3
PP2	-	2.8

[a]The concentration that inhibited enzyme activity by 50%.

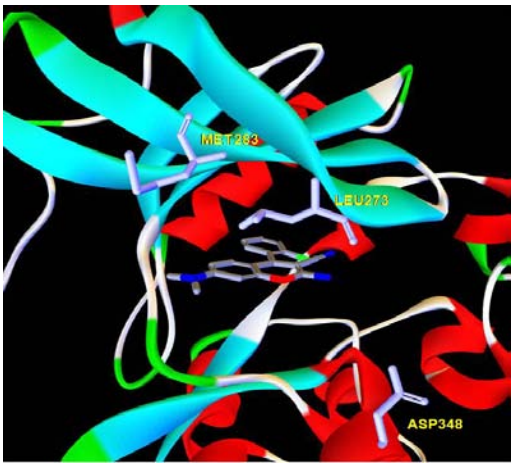


Fig. (5). Structural complex of Src kinase with compound **4c** based on molecular modeling. Compound **4c** and side chains of amino acids are rendered in stick styles. Compound **4c** is in the lowest energy conformer predicted. The carbon skeleton of compound **4c** and side chains are gray, oxygen atoms are red, nitrogens are blue, and the chlorine atom is green. The Figure is drawn using the Accelrys visualization system.

higher inhibitory potency when compared with the 2-methoxysubstituted (**4m**, IC_{50} = 44.6 μM), 2,3-dimethoxy (**4n**, IC_{50} = 45.7 μM), and 2,5-dimethoxy (**4o**, IC_{50} > 150 μM) containing an electron donating methoxy group. Other compounds containing fluorine (**4a**, **4b)**, nitro (**4g**, **4h**) and trifluoromethyl (**4i**) were moderate inhibitors, suggesting that the presence of electron withdrawing groups is preferred for optimal Src kinase inhibitory activity in these analogs. The 2-biphenyl (**4r**), isoquinoline analog (**4s**), and benzodioxolyl analog (**4t**) exhibited moderate to week inhibitory activity (IC_{50} =28.8–30.4 μM).

Compound **4c** (IC_{50} = 11.1 μM) was selected for inhibitory selectivity assays against EGFR, a receptor tyrosine kinase, Csk, a tyrosine kinase that phosphorylates Src, and Lck, a member of Src family kinase. IC_{50} values were >300 μM, 101.7 μM, and 46.8 μM for EGFR, Csk, and Lck, respectively. These data suggest that compound **4c** was relatively selective against Src when compared with the selected kinases (EGFR > Csk > Lck).

Molecular modeling and minimization was used to explore how **4c** would fit within the ATP-binding site of the enzyme (Fig. **5**). The modeling studies indicated that 2-chlorophenyl group in **4c** occupies the hydrophobic binding pocket similar to tolyl group of PP1 with slightly different orientations. The 2-cholorophenyl group contributed to Src kinase inhibitory activity possibly through hydrophobic in-

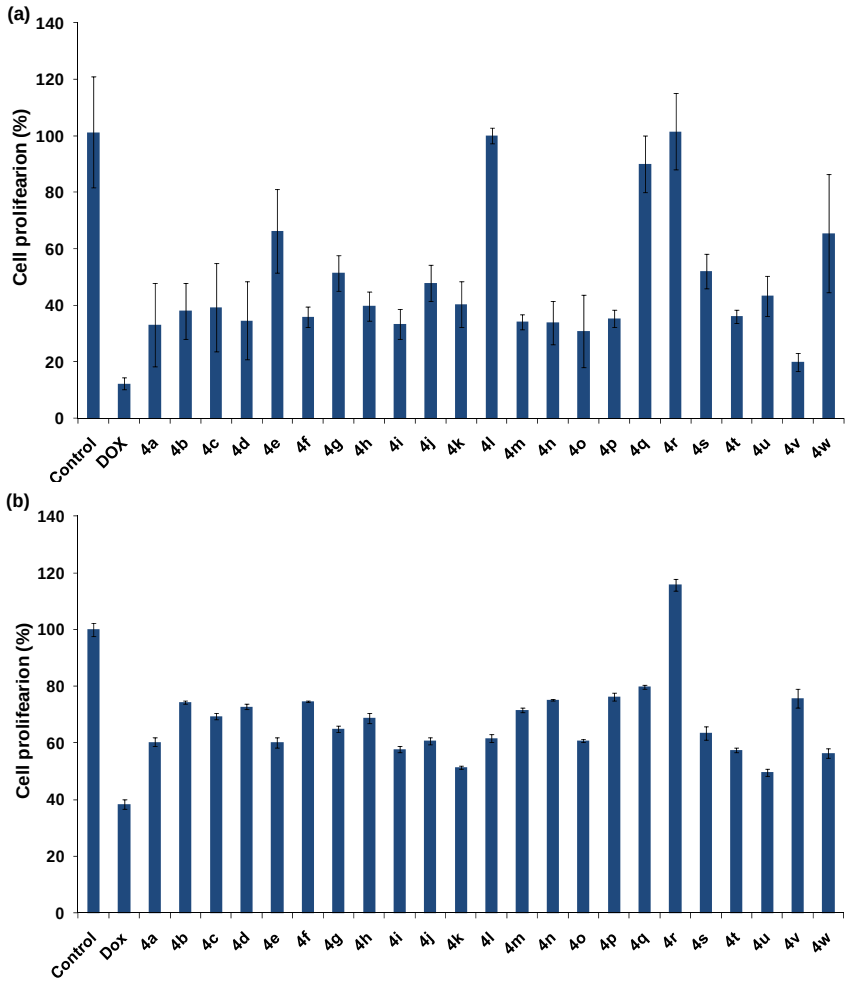


Fig. (6). Inhibition of HT-29 (**a**) and CCRF (**b**) cell proliferation by compounds **4a-w** (50 μM) after 72 h incubation. The results are shown as the percentage of the control DMSO that has no compound (set at 100%). All the experiments were performed in triplicate.

teractions with a large hydrophobic pocket in the ATP binding site. The chromene core mimics the adenine base of ATP and is oriented towards the cavity formed from side chains of helix αC and helix αD. This cavity is normally occupied by carbohydrate moiety followed by triphosphate group of ATP.

Anticancer Activities

The effect of the inhibitors at the concentration of 50 μM on the cell proliferation of human colon adenocarcinoma (HT-29) cancer cells that overexpress c-Src [35] was also evaluated and compared with that of and leukemia (CCRF-CEM) cell line (Fig. **6**). In general, the compounds were more potent against HT-29 that expresses highly activated Src when compared with CCRF-CEM. Compounds **4v** and **4o** inhibited the cell proliferation of colon cancer cells (HT-29) by 80% and 69%, respectively, (Fig **6a**) whereas compounds **4u** and **4k** inhibited the growth of leukemia cancer cells (CCRF-CEM)) by approximately 50% and 49%, respectively (Fig. **6b**).

There were no significant correlation between Src kinase inhibitory potency of the compounds and the growth inhibition of cancer cells since compounds **4c**, **4h**, **4i**, and **4k** were modest Src kinase inhibitors and inhibited HT-29 cell proliferation by 60-67% but compound **4v** that showed high cell proliferation inhibitory effect against colon cancer cells was a weak Src kinase inhibitor (IC_{50} > 300 μM). Substituted 4-aryl-4*H*-chromens-3-carbonitrile have shown to have anticancer activity through apoptosis-inducing effect [23,24]. The data suggest that modest Src inhibition may only one of the mechanisms involved in anticancer activities of these compounds. Furthermore, the correlation between *in vitro* enzymatic and cell-based assays is not always perfect possibly because of the diversity in solubility and cellular uptake of the compounds.

CONCLUSION

A series of 4-aryl substituted derivatives of 2-amino-7-dimethylamino-4*H*-chromene-3-carbonitrile were evaluated for inhibition of Src kinase and cell proliferation in human colon adenocarcinoma (HT-29) and leukemia (CCRF-CEM) cell lines. In summary, structure-activity relationship studies revealed that the incorporation of less bulkier electron withdrawing groups such as chloro, nitro, and trifluoromethyl at positions 2 or 3 of 4-aryl is preferred and well tolerated compared to other groups in generating Src inhibitory activity. The data provide insights for key structural requirements for further optimization of chromene-carbonitrile derivatives as a scaffold and generating more potent and selective Src kinase inhibitors and/or anticancer agents.

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