Synthesis and Biological Evaluation of 3-(Substituted-benzylidene)-1,3-dihydro-indolin Derivatives as Human Protein Kinase CK2 and p60$^{c\text{-}Src}$ Tyrosine Kinase Inhibitors

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Human protein kinase CK2 is an ubiquitous serine/threonine kinase that is typically found in tetrameric complexes consisting of two catalytic (α and/or α') and two regulatory β subunits. Although there is growing evidence that besides the participation of CK2 in a complex series of cellular functions, this protein kinase is involved in cell viability, cell proliferation, and neoplastic transformation. In the present study, a series of 3-(substituted-benzylidene)-1,3-dihydro-indolin-2-thione derivatives and the corresponding indolin-2-one congeners were tested for their inhibition of human recombinant protein kinase CK2 *in vitro*. The efficacy of these compounds was compared with their inhibitory results of p60$^{c\text{-}Src}$ tyrosine kinase. It was found that 3-(substituted-benzylidene)-1,3-dihydro-indolin-2-thione derivatives are more effective than indolin-2-one congeners for the inhibition of CK2 and p60$^{c\text{-}Src}$ tyrosine kinase.

Key words neoplastic transformation; human protein kinase; casein kinase-2 (CK2); p60$^{c\text{-}Src}$; cancer

Protein kinases have been thoroughly investigated as important targets for therapeutic intervention.[1)] Casein kinase-2 (CK2) probably is the most pleiotropic member of the protein kinase family, with >200 substrates known to date.[2)] It now seems unlikely that this enzyme plays any role in the *in vivo* phosphorylation of casein, the protein from which its name originally derived.[3)] Many investigations suggest that CK2 plays an essential role at various stages during the cell cycle. Genetic studies in yeast indicate that CK2 is required through both G1/S and G2/M transitions.[3)] Furthermore, cell cycle progression in mammalian cells can be inhibited by blocking CK2 activity.[4,5)] These studies suggest that CK2 is required at multiple transitions in the cell cycle and could provide a promising target to interrupt the progress of proliferation in eukaryotic cells.[6)] Beyond the importance of CK2 in the context of cell survival and cell proliferation, there is a large body of evidence that CK2 is involved in neoplastic transformation and cancer.[7,8)] In a number of different cancers such as prostate,[9)] mammary gland,[10)] lung[11)] and others,[12)] abnormally high levels of CK2 have been observed. The results of these investigations and others suggest that CK2 is an attractive and promising target for anti-neoplastic therapeutics. CK2 is exploited by viruses to phosphorylate proteins essential to their lifecycle and may also play a role in viral infections. This makes CK2 additionally an attractive target for antiviral drugs.[13)] Despite the growing evidence on CK2 participation in malignant transformation and cancer, only few inhibitors of this enzyme are available.[14)] For long the best known inhibitors were emodin (IC_{50}=2.0 μM)[15)] and 4,5,6,7-tetrabromo-1-*H*-benzotriazole (TBB, 0.9 μM).[16)] More recently, a 7-substituted indoquinazoline compound was identified out of a 400000-compound library with an IC_{50} value of 0.08 μM towards the rat enzyme by using a virtual screening approach.[17)] Moreover, some polyhalogeno benzimidazole compounds have considerable inhibitory activity against CK2 (IC_{50} in the 0.49—0.93 μM range).[18)]

3-(Substituted-benzylidene)-1,3-dihydro indolin-2-one derivatives have been reported as potent and selective inhibitors of different receptor tyrosine kinases. Among them, SU5416[19)] and SU6668[20)] (Fig. 1) were reported as the most active compounds against VEGF-R (Flk-1/KDR). Another compound, Sunitinib Maleate (Fig. 1), was found an inhibitor of multiple receptor tyrosine kinases involved in cancer including VEGF and platelet-derived growth factor receptors.[21)] More recently, 1-benzyl-indole-2-piperidinoethyl carboxylate was found a potent inhibitor of p60$^{c\text{-}Src}$ tyrosine kinase with an IC_{50} value of 1.37 μM.[22)] In our previous study, several 3-(substituted-benzylidene)-1,3-dihydro-indolin-2-one and thione derivatives were reported as p60$^{c\text{-}Src}$ protein tyrosine kinase inhibitors.[23)]

As the part of our interest in the area of protein kinase inhibitors, we synthesized four new 3-(substituted-benzylidene)-1,3-dihydro-indolin-2-one and thione derivatives. These compounds and previously reported 3-(substituted-

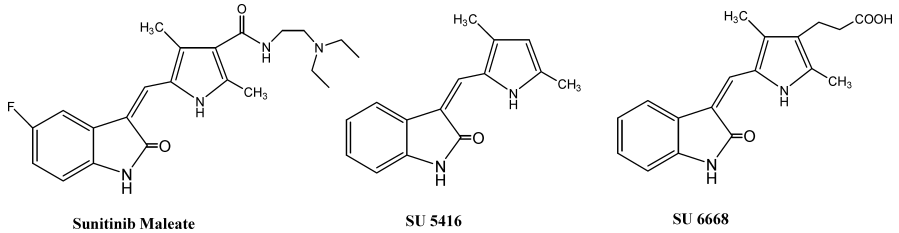

Fig. 1. Structures of the Indole Derivatives Sunitinib Maleate, SU 5416, and SU 6668, Which Are Known as Inhibitors of Human Receptor Tyrosine Kinases

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benzylidene)-1,3-dihydro-indolin-2-one and thione derivatives[23] were tested for their inhibitory activity towards human recombinant protein kinase CK2. Our results indicate that substituted 3-benzylidene-1,3-dihydro-indolin-2-thiones could provide a new molecular scaffold for the rational design of selective human CK2 inhibitors.

MATERIAL AND METHODS

General Experimental Procedure Melting points were measured by capillary melting point apparatus (Buchi SMP 20). Thin-layer chromatography was carried out on aluminum-backed silica gel plates (Merck 60 F_{254}). Flash column chromatography was performed on Merck silica gel (230—400 mesh). ^{1}H-NMR spectra were recorded on Bruker 400 AMX spectrometer at 400 MHz and are referenced to Me_4Si for organic solutions. Mass spectra were taken on a Micromass Autospec high-resolution mass spectrometer. Elemental analyses were obtained from a Leco-932 CHNS-O analyzer.

Reagents: *p*-Chloro-benzaldehyde, 3-fluoro-benzaldehyde, and 4-methoxy-benzaldehyde were purchased from Fluka. Hydrazine hydrate (Merck), hydrogen chloride (Merck), sodium carbonate (Merck), anhydrous sodium sulfate (Merck), ethyl acetate (Merck), hexane (Merck), isatine (Aldrich), anhydrous tetrahydrofurane (Aldrich), piperidine (BDH), and ethanol (Riedel) were used for reactions.

Synthesis of Oxoindole (1) 15 g (0.1 mol) isatine was dissolved in 60 ml (1.2 mol) hydrazine hydrate and refluxed at 140 °C for 4 h. The reaction mixture was poured into ice-cold water and acidified by 6 N HCl. After standing at room temperature for 2 days, 7.5 g of pure oxo-indole crystals was obtained. Yield, 55%. mp 127 °C (lit. 127—129 °C).[24]

Synthesis of Thioindole (2) 26.67 g (0.06 mol) of P_2S_5 and 6.36 g (0.06 mol) of Na_2CO_3 were suspended in 20 ml of anhydrous THF at 0 °C and the mixture was stirred for 20 min. 7 g (0.05 mol) oxoindole **1** was dissolved in 25 ml anhydrous THF and added dropwise to this mixture. This was stirred at 0 °C for 30 min, allowed to adapt to room temperature, and stirred overnight. The reaction mixture was poured into ice-cold water and extracted with ethyl acetate (3× 100 ml). The organic layer was washed with brine (3× 100 ml) and dried over anhydrous Na_2SO_4. Evaporation of the solvent gave the crude compound, which was purified by silica gel column chromatography (hexane : ethylacetate, 70 : 30). By this procedure 6.5 g of pure compound was obtained. Yield 83%. mp 149 °C (lit. 147—149 °C in MeOH).[25]

General Synthesis of 3-(Substituted-benzylidene)-1,3-dihydro-indolin-2-one and 2-Thione Derivatives (3—16) A reaction mixture of oxoindole or thioindole (1 eq), the aldehyde (1.2 eq), and piperidine (0.1 eq) in ethanol (1—2 ml/1 mmol) was stirred at 90 °C for 3—5 h.[26] This was cooled and the solvent removed under vacuum. The precipitate or oily compounds were purified by silicagel column chromatography (hexane : ethylacetate, 50 : 50). The isomers, obtained as a mixture of *E* and *Z*, were separated by the same solvent system on silica gel column chromatography. The data for minor isomers are not reported.

(*Z*)-3-(3′-Fluoro-benzylidene)-1,3-dihydro-indolin-2-one (**7**): ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 10.62 (s, 1H, NH), 7.59 (s, 1H, H-vin), 7.57—7.49 (m, 4H, H-2′, H-4′, H-5′, H-6′), 7.43 (d, 1H, J=7.59 Hz, H-4), 7.31 (t, 1H, H-6), 7.23 (t, 1H, H-5), 6.87 (d, 1H, J=7.72 Hz, H-7). MS *m*/*z*: 239.55 (M^+).

(*E*)-3-(4′-Chloro-benzylidene)-1,3-dihydro-indolin-2-thione (**10**): ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 11.56 (s, 1H, NH), 7.69 (d, 2H, J=7.23 Hz, H-2′, 6′), 7.48—7.12 (m, 3H, H-vin, H-4, H-6), 6.95 (t, 1H, H-5), 6.77—6.63 (m, 3H, H-3′, 5′, H-7). MS *m*/*z*: 272.34 (M+H).

(*Z*)-3-(3′-Fluoro-benzylidene)-1,3-dihydro-indolin-2-thione (**14**): ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 11.59 (s, 1H, NH), 7.72 (t, 1H, H-4′), 7.33 (s, 1H, H-vin), 7.30—7.24 (m, 3H, H-2′, H-5′, H-6′), 7.10 (d, 1H, J=7.92 Hz, H-4), 7.04—6.46 (m, 2H, H-5, H-6), 6.10 (d, 1H, J=7.55 , H-7). MS *m*/*z*: 256.46 (M^+).

(*E*)-3-(4′-Methoxy-benzylidene)-1,3-dihydro-indolin-2-thione (**15**): ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 11.45 (s, 1H, NH), 7.69 (d, 2H, J=7.57 Hz, H-2′, 6′), 7.40—7.22 (m, 2H, H-vin, H-4), 7.15 (t, 1H, H-6), 6.92 (t, 1H, H-5), 6.77—6.58 (m, 3H, H-3′, 5′, H-7), 3.64 (s, 3H, OCH_3). MS *m*/*z*: 267.20 (M^+).

Preparation of Recombinant Human CK2 Enzyme A purification protocol was set up according to the method of Grankowski *et al.*[27] with modifications. For this purpose α-subunit (CSNK2Al) and β-subunit (CSNK2B) of protein kinase CK2 were expressed separately in a bacterial expression system (pT7-7/BL2l(DE3).[28] Freshly transformed bacterial cells were grown overnight at 37 °C until they reached the stationary phase. With the seperate overnight culture for each subunit, CSNK2Al and CSNK2B, 6 l of fresh medium was inoculated and cultivated until an OD_{500} of 0.6 was reached. Protein expression was induced by adding IPTG to a final concentration of 1 mM and was run at 30 °C for 5—6 h for CSNK2Al and at 37 °C for 3 h for CSNK2B. Bacterial cells were harvested by centrifugation (6000×***g*** at 4 °C for 10 min) and disrupted by sonification (3×30 s on ice). Cell debris was removed by centrifugation and the bacterial extracts by this strategy for both subunits were combined and subjected to a three-column purification procedures as described previously.[28] The fractions containing active CK2 holoenzyme were determined by activity measurement using the synthetic peptide substrate RRRDDDSDDD. Fractions exhibiting CK2 enzymatic activity were combined and analyzed by SDS page and western blot. They were stored in aliquots at 80 °C until used for testing.

Inhibition of Recombinant Human CK2 CK2 holoenzyme (50 ng=5 U, 1 U corresponds to the amount of kinase catalyzing the transfer of 1 μmol phosphate/min to the specific substrate) were pre-incubated at room temperature for 10 min with the inhibitor in a final concentration of 10 μM or DMSO as a control in a total volume of 20 μl in kinase buffer (50 mM Tris/HCl, pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT). The inhibitors were routinely kept in a DMSO stock solution with a concentration of 5 mM. The reaction was started by adding 30 μl assay buffer (25 mM Tris/HCl, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 100 μM ATP, 0.19 mM substrate (synthetic peptide RRRDDDSDDD) 0.6 μCi [γ-^{32}]ATP). The synthetic substrate peptide RRRDDDSDDD has been identified in a study on the substrate specificity determinants for CK2, which is the most efficiently phosphorylated peptidic substrate.[29] It is routinely used in studies on the enzyme activity of human CK2.[30] The

arginine residues at the N-terminal end can be used for purification of phosphorylated substrate by ion exchange chromatography and have no negative effect on enzyme activity. Incubation was continued at 37 °C for 15 min then the mixture was spotted onto a P81 ion exchange paper. After washing three times with excess phosphate (85 mM H_3PO_4) and following with ethanol, the filter was dried and the appending radioactivity determined by scintillation counter (Packard). The radioactivity of the DMSO control was set as 100% CK2 activity and the values were obtained with the different inhibitors in 10 μM concentration. Each value was determined at least three times in independent experiments.

RESULTS AND DISCUSSION

The 3-(substituted-benzylidene)-1,3-dihydro-indolin-2-thione and 2-one derivatives **3**—**16** were synthesized by condensing compounds **1**, **2**, and substituted aryl aldehydes in the presence of different bases (Knovenagel reaction)[31] (Chart 1). Although knovenagel reaction was reported for the synthesis of 3-(substituted-benzylidene)-1,3-dihydro-indolin-2-one derivatives, it was successfully applied for the synthesis of 3-(substituted-benzylidene)-1,3-dihydro indolin-2-thione derivatives in our laboratory.[23] Synthesis of oxoindole (**1**) was achieved by a Wolff–Kishner-like reduction of isatin with hydrazine hydrate.[32] The oxoindole was changed to the thioindole (**2**) by P_2S_5 and Na_2CO_3 in THF.[33] All original 3-(substituted-benzylidene)-1,3-dihydro-indolin-2-thione derivatives were first synthesized in our laboratory.[23] The synthesis and analysis data of newly synthesized original 3-(substituted-benzylidene)-1,3-dihydro-indolin-2-thione derivatives **10**, **14**, and **15** are summarized in the experimental section. Among the 3-(substituted-benzylidene)-1,3-dihydro-indolin-2-one derivatives, compounds **7** and **9** are original. The synthesis and analysis data of compound **7** were reported in this paper and compound **9** was reported in our previous publication.[23] Data on other 3-(substituted-benzylidene)-1,3-dihydro-indolin-2-one derivatives were previously reported by Daisley and Walker[34] (compound **3**), Neber and Rocker[35] (**4** and **6**), Howard *et al.*[36] (**5**), and Sun *et al.*[26] (compound **8**).

An assay for inhibitor testing was set up in analogy to Yim *et al.*[15] and Sarno *et al.*[16] with modifications as described in Materials and Methods. The results of the test are summarized in Table 1. None of the indolin-2-one derivatives exhibited significant inhibition of human CK2. However, compounds **8** and **9** showed a slight inhibition of the enzyme. To our surprise, the potency of the identical thione congeners was higher for each example (inhibition in the 10—30% range). This indicates that the thione group plays an important role for binding of the inhibitor to the active site of CK2. Since the enzyme inhibitory potency was found considerable for compounds **12** and **16**, their IC_{50} values were calculated and are reported as 24.6 and 65 μM, respectively, in Table 1.

IC_{50} values were defined as the concentration of a compound required to achieve 50% inhibition of tyrosine phosphorylation on the $p60^{c\text{-}Src}$ tyrosine kinase to ligand-stimulated control reactions in the presence of vehicle alone (dimethyl sulfoxide). Compounds with IC_{50} values >100 μM were considered inactive. Among the tested compounds, **4**, **13**, and **16** were found most active against the $p60^{c\text{-}Src}$ kinase. The maximum inhibition attained among the tested compounds was IC_{50} 21.20 μM for compound **13** as shown in Table 1. Compound **16** showed good activity at the 30.92 μM concentration. The 3-(2′-chloro-benzylidene)-1,3-dihydro-indolin-2-one **4** (70.37 μM) also slightly inhibited the enzyme. Interestingly, 3-(2′-chloro-benzylidene)-1,3-dihydro-indolin-2-one **4** has a 4-fold greater potency than thio congener **11**. Only 3-(3′,4′-dichloro-benzylidene)-1,3-dihydro indolin derivatives of congeners **5** and **12** produced equal activity. Since some thio congeners were found more potent than the oxo, we suggest that the replacement of oxo with a thio group could play a more important role in enhancement of activity. The same substitutions to different congeners have different impacts on activity. However, no clear relationships were observed between the activity profiles of identical congeners. The potency of this chemical series may also depend, to a large extent, on the three-dimensional structure of R-substituents since a wide range of IC_{50} values (from >1000 μM to 20 μM) was observed depending on the nature of R-substituents as shown in Table 1.

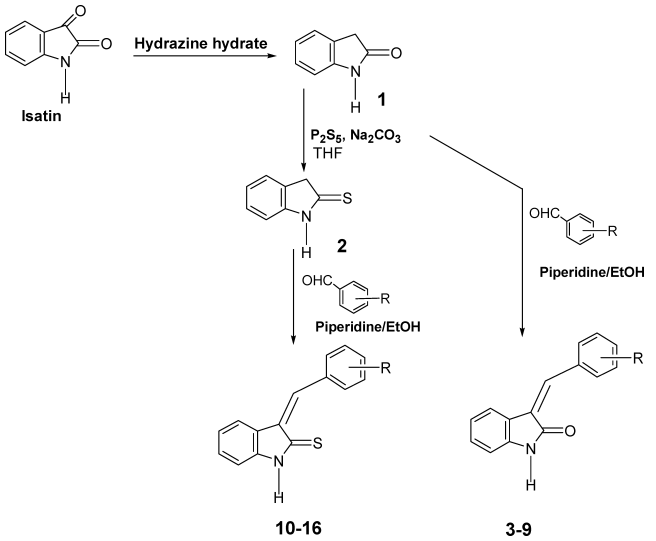
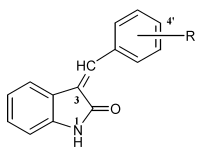


Chart 1. Preparation of Compounds **1**—**16**

The introduction of 3′-OH, 4′-OCH_3 substituents in the aromatic ring (compound **16**) caused higher activity of enzyme CK2 and $p60^{c\text{-}Src}$. In conclusion, the thio derivatives are more effective for inhibition of both enzymes. This suggests that 3-(substituted-benzylidene)-1,3-dihydro-indolin-2-thiones can provide a new structural principle for the rational design of selective human CK2 and $p60^{c\text{-}Src}$ tyrosine kinase inhibitors.

Although we have no experimental evidence as yet on this issue, we propose that our compounds, based on structural similarities to SU5416 and SU6668, bind at the ATP-binding site in a competitive mode. At this stage, this would seem true for both enzymes, and it appears interesting that compound **3** (2-one) showed weak potency on $p60^{c\text{-}Src}$, but no inhibition of CK2, whereas compound **10** (2-thione) showed some inhibition on CK2 but not on $p60^{c\text{-}Src}$. These observations could be the clue for the design of selective inhibitors for both kinases. Such inhibitors could be helpful in the dissection of the different roles of these enzymes in cellular processes such as cell proliferation and signal transduction. It is also known that some compounds considered to bind to the

Table 1. Inhibition of Human Protein Kinase CK2 by 3-(Substituted-benzylidene)-1,3-dihydro-indolin-2-one and Indolin-2-thione Derivatives



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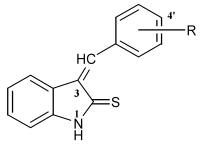

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Compd. No.	R	Isomer	mp (°C)	CK2 Inhib. (%)	CK2 IC_{50} (μM)	$p60^{c\text{-}Src}$ PTK IC_{50} (μM)
3	4′-Cl	*E*	191—193	0		350.0
4	2′-Cl	*Z*	181	0		70.4
5	3′, 4′-Cl	*Z*	183—186	4		149.1
6	2′, 6′-Cl	*Z*	164—167	0		>1000
7	3′-F	*Z*	145—147	0		>1000
8	4′-OCH_3	*E*	156	18		>1000
9	3′-OH, 4′-OCH_3	*Z*	153	23		203.2
10	4′-Cl	*E*	188—189	30		>1000
11	2′-Cl	*Z*	173—174	10		305.6
12	3′, 4′-Cl	*Z*	158—160		24.6	154.5
13	2′, 6′-Cl	*Z*	155—156	19		21.2
14	3′-F	*Z*	226—227	21		>1000
15	4′-OCH_3	*E*	170—171	30		>1000
16	3′-OH, 4′-OCH_3	*E*	95—96		65	30.9

substrate site show considerable selectivity among different enzymes. This gives rise to the possibility that highly specific tyrosine kinase inhibitors can be developed. On the other hand, dual inhibitors of both kinases, *e.g.* by compounds **12** and **16**, could be promising lead candidates for the development of potent anti-tumor drugs.

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