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Short communication

Synthesis, in vitro antiproliferative activities, and Chk1 inhibitory properties of indolylpyrazolones and indolylpyridazinedione

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Abstract

The synthesis of 5-indolylpyrazol-3-one, 4-indolylpyrazol-3-one and 4-indolyl-pyridazin-3,6-dione is reported. Their Chk1 inhibitory properties have been evaluated and their in vitro antiproliferative activities toward three tumor cell lines: murine leukemia L1210, human colon carcinoma HT29 and HCT116 have been determined. 4-Indolyl-pyridazin-3,6-dione is inactive against Chk1 and exhibits weak cytotoxicities toward the tumor cell lines tested. The IC_{50} values toward Chk1 of the two indolylpyrazolones are identical and are in the micromolar range, but the cytotoxicities of 4-indolylpyrazol-3-one are significantly stronger than those of 5-indolylpyrazol-3-one. Since 4-indolylpyrazol-3-one and 5-indolylpyrazol-3-one can present several conformers and tautomeric forms, molecular modelling in the ATP binding site of Chk1 has been carried out to investigate which form could induce the best stabilization in the active site of the enzyme. To get an insight into the kinase selectivity of these compounds, their inhibitory activities toward Src kinase were evaluated.

Keywords: Indolylpyrazolones; Indolyl-pyridazin-3,6-dione; Antitumor agents; Chk1 inhibitors

1. Introduction

The pyrazol-3-one heterocycle is found in many biologically active compounds. Some of them are inhibitors of tumor necrosis factor-α production and of the JNK3 kinase [1].

Others were found to be antihyperglycemic agents and were isolated as their hydroxy tautomers [2]. Very recently, moderate in vitro antiproliferative activities were described for *N*,*N*-dialkylaminoalkyl substituted bisindolyl and diphenyl pyrazolone derivatives [3]. In the search for new Chk1 inhibitors, we were interested in 5-indolylpyrazol-3-one and 4-indolylpyrazol-3-one (Fig. 1). We report here their synthesis together with their Chk1 inhibitory activities and their cytotoxicities toward three tumor cell lines: murine leukemia L1210, human colon carcinoma HT29 and HCT116.

Staurosporine isolated from cultures of *Streptomyces* sp. is a non-selective kinase inhibitor [4]. Among the kinases that are inhibited by staurosporine, Chk1, which is a serine/threonine kinase, plays a major role in the cell cycle arrest in response to DNA damage [5].

Staurosporine is an ATP-competitive Chk1 inhibitor. In the structure of Chk1 in complex with staurosporine, two hydrogen bonds are observed between the lactam heterocycle and the ATP binding site of the enzyme: the first one between the lactam NH and the carbonyl oxygen of Glu^{85}, and the second one between the oxygen of the carbonyl group of the lactam function of the drug and the amide nitrogen of Cys^{87} [6]. The structures of both 4-indolylpyrazol-3-one and 5-indolylpyrazol-3-one could be compatible with the formation of these hydrogen bonds, the indole moiety being oriented either toward the carbonyl group or

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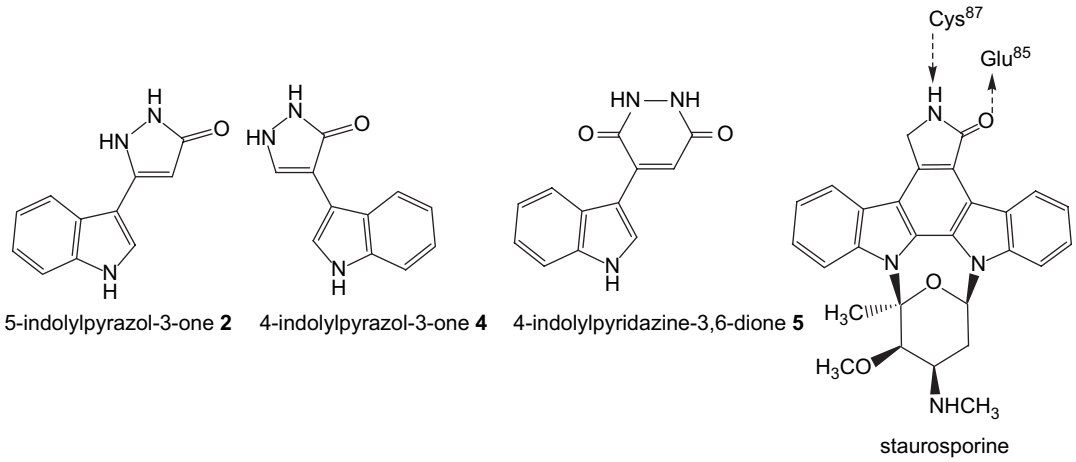


Fig. 1. Structures of 5-indolylpyrazol-3-one, 4-indolylpyrazol-3-one, 4-indolylpyridazine-3, 6-dione and staurosporine.

toward the opposite site like the two indole moieties of staurosporine. Therefore, in spite of the structures of our pyrazolones, less rigid than staurosporine and not necessarily planar, the inhibitory activities toward Chk1 have been investigated.

To get an insight into the influence of the size of the upper heterocycle, indolyl-pyridazin-3,6-dione was synthesized and its biological properties were evaluated and compared with those of the indolylpyrazolones. These compounds can present tautomeric forms that could bind differently in the ATP binding site of Chk1. Accordingly, molecular modelling has been carried out to get an insight into the stabilization of the different forms in the active site of Chk1. Since, very recently several pyrazolones were described as potent Src kinase inhibitors [7], the inhibitory activities of 4-indolylpyrazol-3-one, 5-indolylpyrazol-3-one and 4-indolyl-pyridazin-3,6-dione toward Src were also evaluated.

2. Results and discussion

2.1. Chemistry

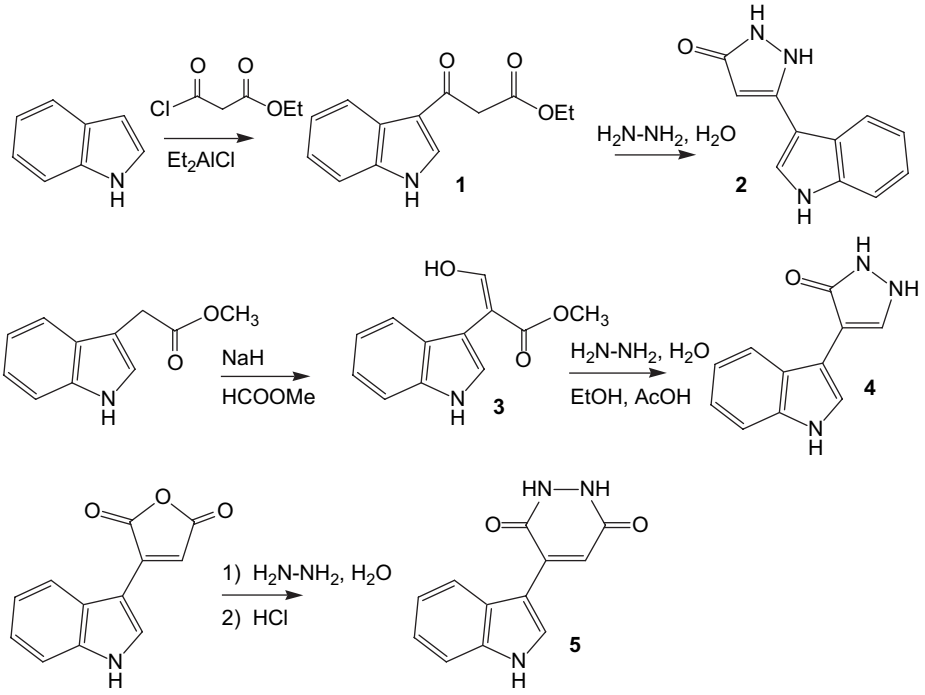
The synthetic method for the preparation of compound **2** is outlined in Scheme 1. Ethyl 1-chloro-malonate was coupled to indole in the presence of diethylaluminium chloride. This Lewis acid was successfully used for the acylation of indoles in high yields [8]. Reaction of the resulting ethyl 3-oxo-3-(1*H*-indol-3-yl)-propionate **1** with hydrazine hydrate according to a method described for the preparation of polysubstitued pyrazolones [9] led to pyrazolone **2**. Like pyrazol-3-ones without substituents on the nitrogens, compound **2** could exist as three tautomers **2**, **2′** and **2″** (Scheme 2) [10]. In the ^{1}H NMR spectrum of compound **2** in DMSO, the two signals observed for the exchangeable protons of the pyrazolone heterocycle are broad signals at 9.90 and 11.53 ppm. However, the ^{1}H NMR spectrum does not allow to confirm the presence of tautomeric forms.

For the two-step synthesis of compound **4**, formylation of commercial methyl 3-indolyl-acetate with methyl formate after deprotonation with sodium hydride according to a method described with a phenyl substituent instead of an indole [11] led to 3-hydroxy-acrylate **3** in a quantitative yield. The formyl form was not observed in closely related 3-hydroxy-acrylates [12,13]. Reaction of compound **3** with hydrazine hydrate in an acidic medium as described in non-aromatic series [14] gave pyrazolone **4** in 74% yield. As for compound **2**, the two exchangeable protons of the pyrazolone **4** are broad signals shifted at 10.00 and 11.36 ppm. The ^{1}H NMR spectrum does not allow to confirm a tautomeric equilibrium between **4**, **4′** and **4″**. Compound **4** has also been obtained previously in two steps from ethyl 3-indoleacetate via 3-dimethylamino-2-(1*H*-indol-3-yl)propenoate [15].

4-(Indol-3-yl)-pyridazine-3,6-dione **5** (Scheme 1) was prepared by the reaction of hydrazine hydrate with 3-(indol-3-yl)-furane-2,5-dione according to a method described in indolocarbazole series [16], followed by acidic treatment. In DMSO, the exchangeable protons gave three thin signals at 10.58, 11.74 and 12.00 ppm. Two isomers could be obtained in this reaction: a five-membered ring *N*-amino derivative [17–19] or a six-membered ring hydrazide [19,20]. In a five-membered ring *N*-amino derivative, the two protons of the amino group should be shifted at about 5 ppm. The absence of exchangeable protons at about 5 ppm confirms unequivocally the hydrazide form.

2.2. Chk1 inhibitory activities

To our knowledge, compound **4** has never been tested toward kinases and only compounds bearing pyrazolinone heterocycles different than the ones present in compound **2** are claimed in two patents as serine/threonine and tyrosine kinase inhibitors [21,22]. In Schemes 2A and 3A are shown the tautomeric forms of compounds **2** and **4** together with the possible fundamental hydrogen bonds with Glu^{85} and Cys^{87} in the ATP binding site of Chk1. Conformers **2′a** (conformer of **2′**), and **4′a** (the same molecule as **4′** but with a rotation of 180°) as well as **2″** and **4″** may be able to form similar hydrogen bonds with Glu^{85} and Cys^{87}. These hydrogen bonds are also possible in compound **5** (Scheme 4A). Molecular modelling has been carried out using as model the complex structure of Chk1/staurosporine [6] downloaded from the Protein Data Bank. Concerning



Scheme 1. Synthetic schemes for compounds **2**, **4**, and **5**.

compound **2** (Scheme 2B), the same hydrogen bond net as observed with staurosporine is only conserved with the carbonyl form **2**. Conformers **2′** and **2′a** show only one hydrogen bond between the hydroxy group and the carbonyl of Glu[85], whereas with **2″** two hydrogen bonds are observed but both with the hydroxy group. With compound **4** (Scheme 3B) molecular modelling gave similar results. Only compound **4** gives the same hydrogen bond net as that observed with staurosporine. With **4′** and **4′a**, only one hydrogen bond is formed with Glu[85], whereas with **4″** two hydrogen bonds are observed but both with Glu[85]. Finally, with compound **5** (Scheme 4B) only one hydrogen bond can be observed with Glu[85].

The Chk1 inhibitory activities of compounds **2**, **4** and **5** were evaluated (Table 1). The IC_{50} values are identical for compounds **2** and **4** (5 μM) suggesting that the two hydrogen bonds with Glu[85] and Cys[87] are conserved. Compound **5** was inactive toward Chk1, which is in agreement with the molecular modelling results. The biological data suggest that a five-membered ring upper heterocycle is necessary for Chk1 inhibition.

2.3. Src kinase inhibitory activities

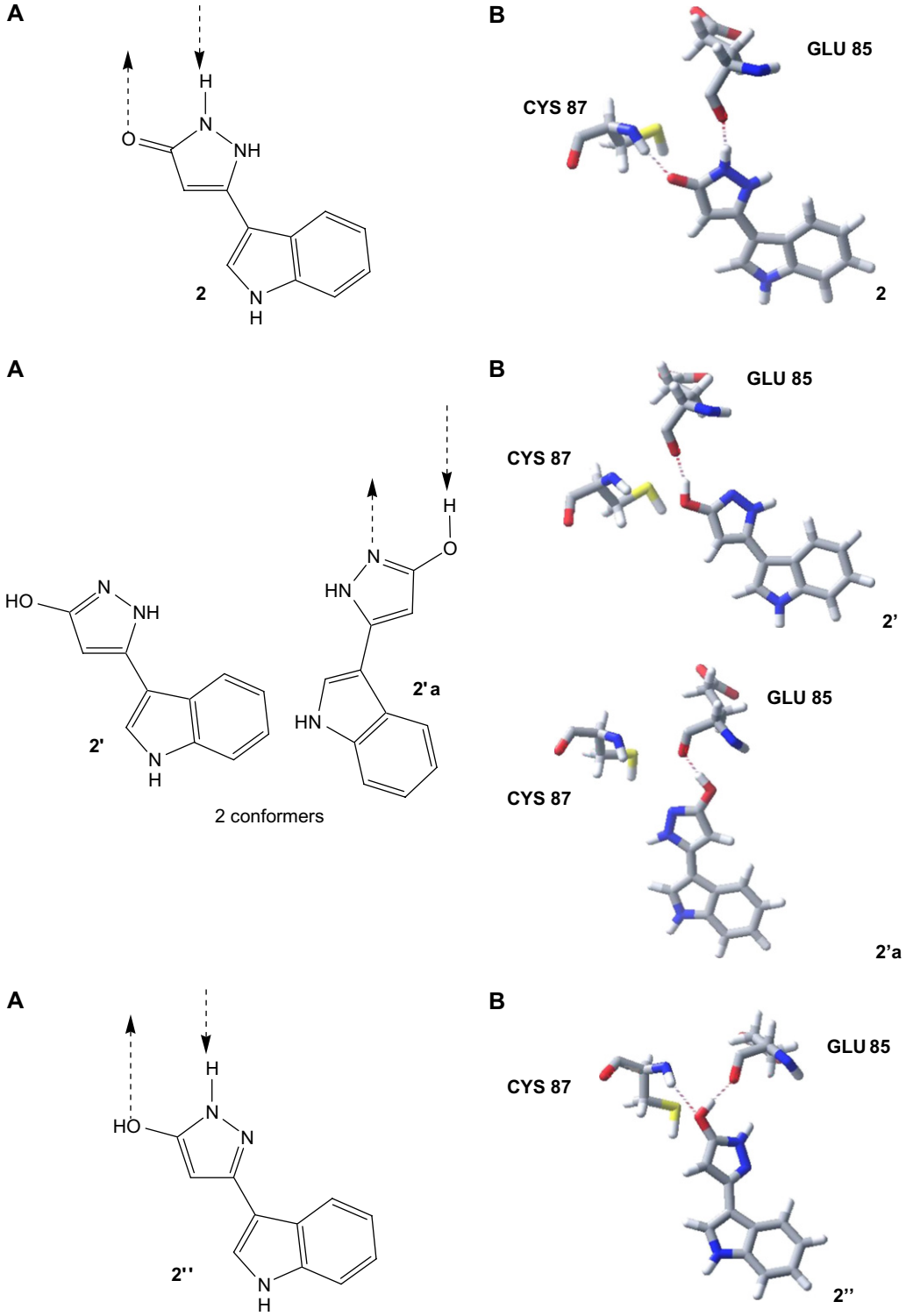
To get a first insight into the kinase selectivity, the inhibitory activities of compounds **2**, **4** and **5** were evaluated toward the tyrosine kinase Src. Indeed, a recent paper [7] reports Src inhibitory activities in the micromolar range of some pyrazolone derivatives in which the carbonyl group and the adjacent NH of the pyrazolones show hydrogen bond interactions with the Glu[339] and Met[341] residues of Src. The percentages of Src inhibition at a drug concentration of 10 μM were determined (Table 1). None of the three compounds inhibits Src significantly.

2.4. In vitro antiproliferative activities

The cytotoxicities of compounds **2** and **4** toward three tumor cell lines: murine leukemia L1210, human colon carcinoma HT29 and HCT116 were determined (Table 2). The most sensitive cells are HCT116 colon carcinoma cells. Compound **4** is much more cytotoxic than compound **2** against the three tumor cell lines tested. The inhibition of Chk1 is not expected by itself to lead to cytotoxic compounds. However, the ATP binding site being similar in all the kinases, except the surrounding pockets, a Chk1 inhibitor generally inhibits other kinases which could be responsible for its cytotoxicity. Probably, the inhibition of targets other than Chk1 can explain the cytotoxicity of compound **4**. The in vitro antiproliferative activities observed for compound **5** are very weak, this compound is very likely a poor kinase inhibitor.

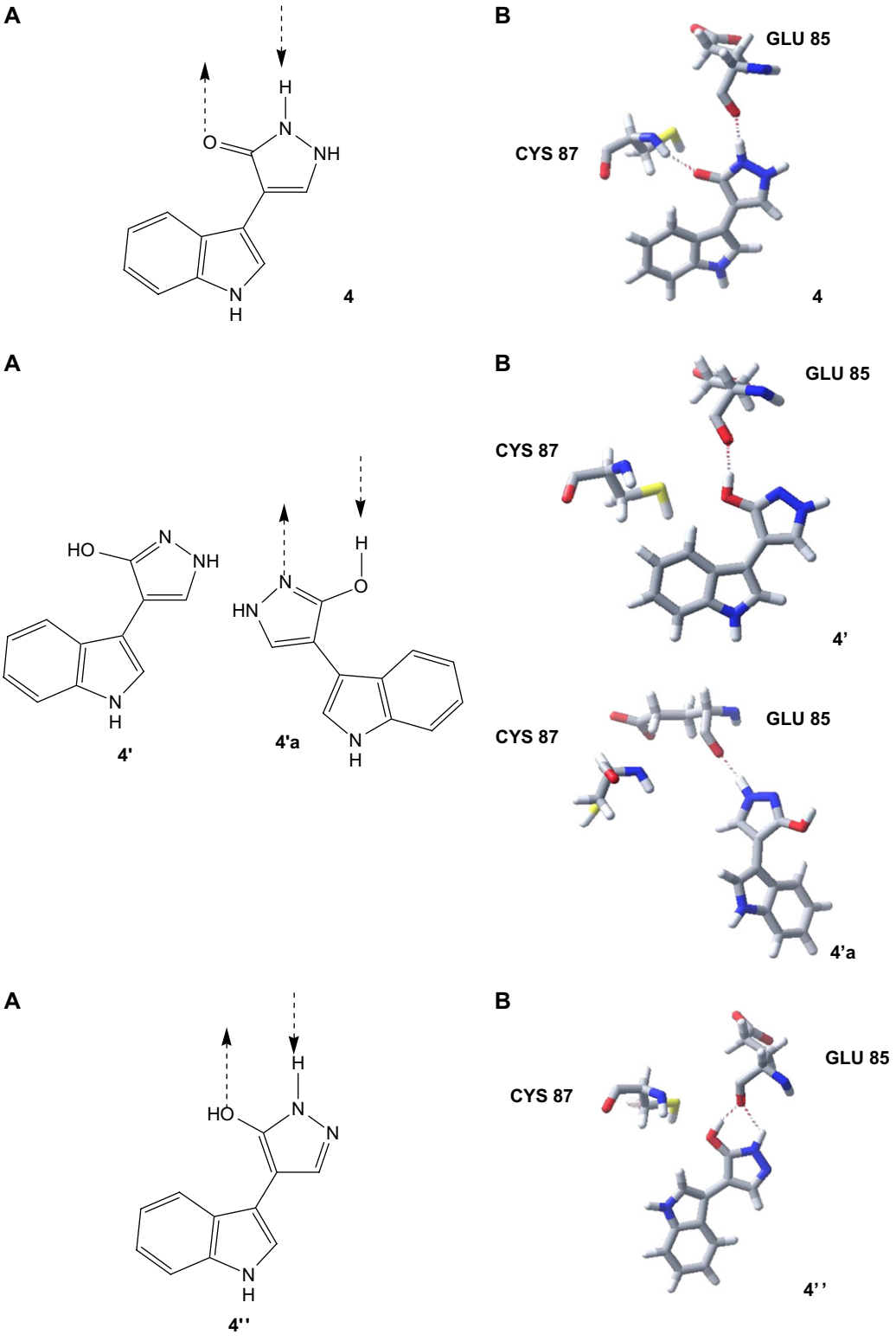
3. Conclusion

In conclusion, this work reports the synthesis of 4-indolylpyrazol-3-one and 5-indolylpyrazol-3-one, their cytotoxicities toward three murine and human tumor cell lines and their inhibitory potencies toward Chk1. In spite of the position of the indole substituent in 4 or 5 of the pyrazolone moiety, the Chk1 inhibitory potencies are identical, suggesting an identical orientation of the pyrazolone heterocycle within the ATP binding pocket of Chk1. The differences observed in the cytotoxicities suggest targets other than Chk1 for compound **4**. These targets

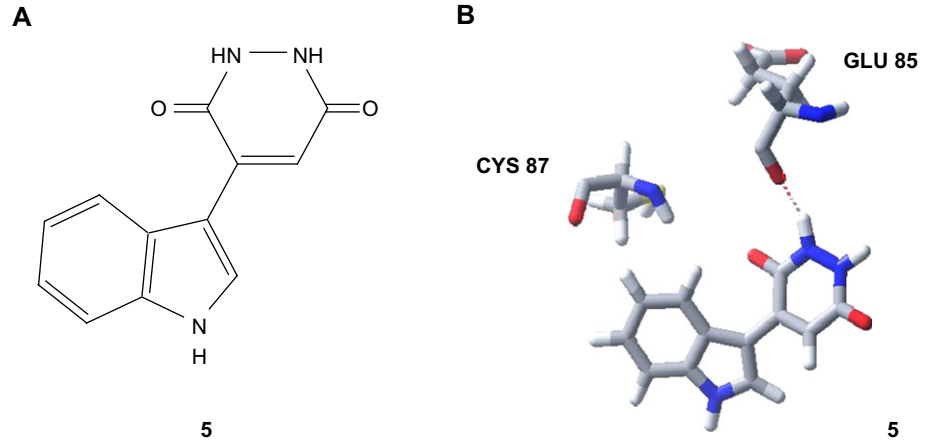


Scheme 2. On the left, tautomeric forms for compound **2** are presented with possible hydrogen bonds between Glu[85] and Cys[87] residues in the ATP binding site of Chk1; on the right, hydrogen bonds obtained after molecular modelling are presented.

could be various kinases. The inhibitory activities of compounds **2**, **4** and **5** have been evaluated toward the tyrosine kinase Src, but none of the three compounds inhibits this tyrosine kinase significantly. A recent work describes a buried pocket in Chk1 at the periphery of the ATP binding site which can be used for the conception of potent and selective Chk1 inhibitors [23]. In particular, Leu[84] and Asn[59] residues can be exploited to form possible supplementary hydrogen bonds with substituents introduced on the indole moiety of compound **2**. We are now investigating this hypothesis. The poor Chk1



Scheme 3. On the left, tautomeric forms for compound **4** are presented with possible hydrogen bonds between Glu[85] and Cys[87] residues in the ATP binding site of Chk1; on the right, hydrogen bonds obtained after molecular modelling are presented.



Scheme 4. After molecular modelling, only one hydrogen bond is observed between compound **5** and Glu^{85} residue in the ATP binding site of Chk1.

inhibitory properties of indolylpyridazin-3,6-dione seem to indicate that a five-membered ring upper heterocycle is necessary for an efficient Chk1 inhibition.

4. Experimental section

4.1. Chemistry

IR spectra were recorded on a Perkin–Elmer 881 spectrometer (ν in cm^{-1}). NMR spectra were performed on a Bruker AVANCE 400 (chemical shifts δ in parts per million, the following abbreviations are used: singlet (s), broad singlet (br s), doublet (d), doubled doublet (dd), triplet (t), doubled triplet (dt), multiplet (m), quadruplet (q), tertiary carbons (C tert), quaternary carbons (C quat)). Low resolution mass spectra (ESI+) and HRMS were determined on an MS Hewlett Packard engine. Chromatographic purifications were performed by flash silicagel Geduran SI 60 (Merck) 0.040–0.063 mm column chromatography.

*4.1.1. Ethyl 3-oxo-3-(1H-indol-3-yl)-propionate **1***

To a solution of indole (500 mg, 4.27 mmol) in CH_2Cl_2 (20 mL) at 0 °C was added $AlClEt_2$ (820 μL, 964 mg, 6.40 mmol). The mixture was stirred at 0 °C for 30 min. A 1 M solution of ethyl 1-chloro-malonate in hexane (6.50 mL) was added dropwise at 0 °C. After stirring at 0 °C for 2 h, water (20 mL) was added. After extraction with EtOAc, the organic phase was dried over $MgSO_4$ and the solvent was removed. The residue was purified by flash chromatography (eluent EtOAc/C_6H_6 from 1:9 to 4:6) to give compound **1** (370 mg, 1.60 mmol, 38% yield) as a white solid. Mp. 37–39 °C. IR (KBr) $\nu_{C=O}$ 1740 cm^{-1}, ν_{NH} 3220 cm^{-1}. Mass (ESI+) $[M+Na]^+$ 254. ^1H NMR (400 MHz, $CDCl_3$): 1.13 (3H, t, $J = 7.0$ Hz), 3.77 (2H, s), 4.08 (2H, q, $J = 7.0$ Hz), 7.15 (1H, dt, $J_1 = 7.0$ Hz, $J_2 = 1.5$ Hz), 7.18 (1H, dt, $J_1 = 7.0$ Hz, $J_2 = 1.5$ Hz), 7.33 (1H, dd, $J_1 = 7.0$ Hz, $J_2 = 2.0$ Hz), 7.75 (1H, d, $J = 3.5$ Hz), 8.27 (1H, dd, $J_1 = 7.0$ Hz, $J_2 = 1.5$ Hz), 9.96 (1H, s). ^{13}C NMR (100 MHz, $CDCl_3$): 14.1 (CH_3), 47.1 (CH_2), 61.5 (CH_2O), 111.9, 122.1, 122.9, 123.9, 133.3 (C tert arom), 117.1, 124.7, 136.6 (C quat arom), 168.4, 187.4 (C=O).

*4.1.2. 5-(3-Indolyl)-1,2-dihydro-1H,2H-pyrazol-3-one **2***

To a solution of hydrazine hydrate (223 μL, 4.68 mmol) in ethanol (1 mL) heated to reflux was added dropwise a solution of compound **1** (50 mg, 0.216 mmol) in ethanol (2.5 mL). The mixture was refluxed for 24 h. After evaporation, water was added. After extraction with EtOAc, the organic phase was dried over $MgSO_4$ and the solvent was removed. CH_2Cl_2 was added to the solid residue. After filtration, the residue was washed with CH_2Cl_2 to give **2** (20 mg, 0.100 mmol, 46% yield) as a white solid. Mp. 148–149 °C. IR (KBr) $\nu_{C=O}$ 1670 cm^{-1}, ν_{NH} 3100–3600 cm^{-1}. HRMS (ESI+) $[M+H]^+$ calcd for $C_{11}H_{10}N_3O$: 200.0824, found 200.0832. ^1H NMR (400 MHz, DMSO-d_6): 5.82 (1H, s), 7.14 (1H, t, $J = 7.0$ Hz), 7.17 (1H, t, $J = 7.0$ Hz), 7.46 (1H, d, $J = 8.0$ Hz), 7.71 (1H, d, $J = 2.0$ Hz), 7.83 (1H, d, $J = 8.0$ Hz), 9.90 (1H, br s), 11.34 (1H, s), 11.53 (1H, br s). ^{13}C NMR (100 MHz, DMSO-d_6): 86.5, 111.8, 119.3, 119.6, 121.6, 122.9 (C tert), 106.2, 124.4, 136.2, 138.9 (C quat), 161.3 (C=O).

Table 1
IC_{50} values (μM) toward Chk1, percentages of Src inhibition at a drug concentration of 10 μM, and in vitro antiproliferative activities against three tumor cell lines: murine leukemia L1210, human HT29 and HCT116 colon carcinoma (IC_{50} μM)

Compounds	IC_{50} Chk1 (μM)	Percentage of Src inhibition at a drug concentration of 10 μM	L1210	HCT116	HT29
2	5	7.5	49.1	40.4	61.4
4	5	5.5	8.2	2.2	18.4
5	Inactive	0	74.3	60.1	84.3

Table 2
IC_{50} values (μM) toward Chk1 and in vitro antiproliferative activities against three tumor cell lines: murine leukemia L1210, human HT29 and HCT116 colon carcinoma (IC_{50} μM)

Compounds	IC_{50} Chk1 (μM)	L1210	HCT116	HT29
2	5	49.1	40.4	61.4
4	5	8.2	2.2	18.4
5	Inactive	74.3	60.1	84.3

4.1.3. Methyl 3-hydroxy-2-indolyl-acrylate **3**

A solution of methyl 3-indolyl-acetate (100 mg, 0.53 mmol) in methyl formate (1.08 mL, 17.5 mmol) was added to NaH (60% in oil, 103 mg, 2.65 mmol) in Et_2O (2 mL). The mixture was stirred at room temperature for 24 h. MeOH (1 mL) was added to eliminate the excess of NaH, then 50% aqueous acetic acid (2 mL) was added. After extraction with EtOAc, the organic phase was washed with saturated aqueous $NaHCO_3$ then was dried over $MgSO_4$ to give **3** (114 mg, 0.53 mmol, quantitative yield) as an orange solid. Mp. 70 °C. IR (KBr) ν_{CO} 1652 cm^{-1}, ν_{CH} 2924–2855 cm^{-1}, ν_{NH} 3401 cm^{-1}. Mass (ESI+) $[M]^+$ 217, $[M+Na]^+$ 240, $[M+K]^+$ 256. ^1H NMR (400 MHz, DMSO-d_6): 3.59 (3H, s), 6.92 (1H, dt, $J_1 = 8.0$ Hz, $J_2 = 1.0$ Hz), 7.03 (1H, dt, $J_1 = 8.0$ Hz, $J_2 = 1.0$ Hz), 7.23 (1H, d, $J = 2.5$ Hz), 7.27 (1H, d, $J = 8.0$ Hz), 7.34 (1H, d, $J = 8.0$ Hz), 7.89 (1H, s), 10.62 (1H, br s), 11.01 (1H, s). ^{13}C NMR (100 MHz, DMSO-d_6): 50.8 (CH_3), 102.7, 106.6, 125.2, 135.7 (C quat), 111.2, 118.2, 120.3, 120.5, 124.3, 155.1 (C tert), 168.6 (C=O).

4.1.4. 2H,3H-4-(1H-Indol-3-yl)-pyrazolin-3-one **4**

A solution of hydrazine hydrate (4 mL) in a mixture of acetic acid/MeOH (10 mL, 1:1 v/v) was heated to reflux. A solution of compound **3** (570 mg, 2.62 mmol) in acetic acid (5 mL) was added dropwise. The mixture was refluxed for 24 h. After evaporation, water was added to the solid residue, the mixture was filtered off and the residue was washed with EtOAc to give **4** (386 mg, 1.94 mmol, 74% yield) as a white solid. Mp. 240 °C. IR (KBr) $\nu_{C=O}$ 1670 cm^{-1}, ν_{NH} 3395 cm^{-1}. HRMS (ESI+) $[M+H]^+$ calcd for $C_{11}H_{10}N_3O$: 200.0824, found 200.0838. ^1H NMR (400 MHz, DMSO-d_6): 7.06 (1H, t, $J = 7.0$ Hz), 7.13 (1H, t, $J = 7.0$ Hz), 7.41 (1H, t, $J = 8.0$ Hz), 7.56 (1H, s), 7.82 (1H, d, $J = 8.0$ Hz), 7.92 (1H, s), 10.00, 10.98, 11.36 (3H, 3br s, exchangeable protons). ^{13}C NMR (100 MHz, DMSO-d_6): 99.5, 106.9, 125.3, 135.9 (C quat), 111.3, 118.6, 119.7, 120.9, 121.5, 135.9 (C tert), 158.3 (C=O).

4.1.5. 4-(1H-Indol-3-yl)-1H,2H-3,6-dihydro-pyridazin-3,6-dione **5**

A solution of 3-(indol-3-yl)-furane-2,5-dione (100 mg, 0.46 mmol) in hydrazine hydrate (25 mL) was stirred at 60 °C for 17 h. Water (10 mL) was added, then 12 N HCl (6 mL) was added dropwise. After stirring for 30 min at room temperature, then extraction with EtOAc, the organic phase was dried over $MgSO_4$. The solvent was removed and the residue was purified by flash chromatography (eluent: EtOAc 100% to EtOAc/MeOH 90:10) to give **5** (21 mg, 0.09 mmol, 20% yield) as a yellow solid. Mp. 45 °C. IR (KBr) $\nu_{C=O}$ 1651 cm^{-1}, ν_{NH} 3000–3396 cm^{-1}. HRMS (ESI+) $[M+H]^+$ calcd for $C_{12}H_{10}N_3O_2$: 228.0773, found 228.0786. ^1H NMR (400 MHz, DMSO-d_6): 7.19 (1H, t, $J = 7.5$ Hz), 7.21 (1H, t, $J = 7.5$ Hz), 7.23 (1H, s), 7.51 (1H, d, $J = 7.0$ Hz), 7.92 (1H, d, $J = 7.0$ Hz), 8.64 (1H, s), 10.58 (1H, s, NH), 11.74 (1H, s, NH), 12.00 (1H, s, NH). ^{13}C NMR (100 MHz, DMSO-d_6): 112.4, 116.0, 119.6, 120.7, 122.0, 130.8 (C tert), 124.9, 136.4, 136.5 (C quat), 153.6, 158.6 (C=O).

4.2. Chk1 inhibitory assays

Human Chk1 full-length enzyme with an *N*-terminal GST sequence was either purchased from Upstate Biochemicals (no. 14-346) or purified from extracts of Sf9 cells infected with a baculovirus encoding GST-Chk1. Assays for compound testing were based upon the method described by Davies et al. [24].

4.3. Src inhibitory assays

Inhibitors were diluted with a Tecan Evo150 robot. The kinase assay was performed with 4 μL of inhibitor (10%DMSO), 10 μL of kinase assay buffer 4× concentrated (80 mM $MgCl_2$, 200 mM HEPES, 0.4 mM EDTA, 2 mM DTT), 10 μL of substrate peptide (KVEKIGEGYYGVVYK, 370 nM) and 6 μL of Src kinase (stock GTP purified diluted with 1× kinase assay buffer to 200 nM). Ten microlitres of co-substrate (40 μM ATP with 0.2 μCi P^{33}-γ-ATP) was added with a Precision 2000 (Biotek Robotic). The assay mixture was incubated for 20 min at 30 °C and then the reaction was stopped by adding 200 μL of 0.85% orthophosphoric acid, and then transferred to a phosphocellulose filter microplate (Whatman − P81). The plate was washed 3 times with 200 μL of 0.85% orthophosphoric acid and dried with 200 μL of acetone. The remaining activity is measured on a Topcount with 25 μL of scintillation solution (Packard UltimaGold).

4.4. Growth inhibition assays

Tumor cells were provided by American Type Culture Collection (Frederik, MD, USA). They were cultivated in RPMI 1640 medium (Life Science Technologies, Cergy-Pontoise, France) supplemented with 10% fetal calf serum, 2 mM of L-glutamine, 100 units/mL of penicillin, 100 μg/mL of streptomycin, and 10 mM of HEPES buffer (pH = 7.4). Cytotoxicity was measured by the microculture tetrazolium assay as described in Ref. [25]. Cells were continuously exposed to graded concentrations of the compounds for four doubling times, then 15 μL of 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide were added to each well and the plates were incubated for 4 h at 37 °C. The medium was then aspirated and the formazan solubilized with 100 μL of DMSO. Results are expressed as IC_{50}, concentration which reduced by 50% the optical density of treated cells with respect to untreated controls.

4.5. Molecular modelling

All molecular mechanics calculations were performed by the Macromodel [26] molecular modelling software. We used as model the complex structure of CHK1/STAUROSPORINE [6] downloaded from the Protein Data Bank (1NVR file).

Energy minimisation was done with AMBER force field [27,28] using the Truncated Newton Conjugate Gradient method (TNCG).

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