Novel 1H-Pyrazole-3-carboxamide Derivatives: Synthesis, Anticancer Evaluation and Identification of Their DNA-Binding Interaction

Yi Lu, Ting Ran, Guowu Lin, Qiaomei Jin, Jianling Jin, Hongmei Li, Hao Guo, Tao Lu, and Yue Wang

State Key Laboratory of Natural Medicines, China Pharmaceutical University; Laboratory of Molecular Design and Drug Discovery, School of Sciences, China Pharmaceutical University; Nanjing 210009, P. R. China; and Department of Structural Biology, University of Pittsburgh School of Medicine; Pittsburgh, PA 1526, U.S.A.; and State Key Laboratory of Coordination Chemistry, School of Chemistry and Chemical Engineering, Nanjing University; Nanjing 210093, P. R. China.

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Off-target effects, commonly emerged in anticancer drug discovery, often leads to undesired side effects. However, it may also lead to favorable pharmacological profiles in drug re-purposing. In kinase-targeted drug discovery, it has been verified that small molecule inhibitors usually bind to multiple targets, including the rarely reported nucleic acid such as double-strand DNA exemplified by flavopiridol. The off-target effects of kinase inhibitors have always focused on homologous kinases in a dysregulated signaling pathway while rarely on nucleic acid targets at the preliminary stage, although DNA binding affinities, at the late-stage, may be revealed in multitarget small-molecule antibiotic and anticancer drug study. When small molecules bind to the targeted DNA, they alter the normal activity of the DNA with consequent cytotoxicity, cell death, and the abnormal expression of oncopgenes.

Intercalation, alkylation and groove binding are three main mechanisms for compounds targeting DNA. The intercalation often causes obvious conformational changes of DNA and it occurs non-selectively in different DNA binding sites. For the intercalator, improving molecular planarity would facilitate its embedding to DNA base pairs. Alkylation of DNA is the intercalator, improving molecular planarity would facilitate its embedding to DNA base pairs. Alkylation of DNA is the intercalator, improving molecular planarity would facilitate its embedding to DNA base pairs.

Four novel 1H-pyrazole-3-carboxamide derivatives were synthesized, and their antiproliferative effect on cancer cells, kinase inhibition, and in particular, the DNA-binding interaction were investigated to interpret the antitumor mechanisms. A DNA minor groove binding model was developed, and the binding energy was predicted for the compounds. In consistence with the prediction, the binding ability was determined by the electronic absorption spectroscopy under physiological conditions for the compounds, and further verified by viscosity measurement. One compound 5-(3-cyclopropylureido)-N-[4-[1-(4-methylpiperezin-1-yl)methylphenyl]-1-H-pyrazole-3-carboxamide (pym-5) exerted the highest DNA-binding affinity (K_{d} = 1.06 \times 10^{5} M^{-1}). And it demonstrated more than 50% decrease of the emission intensity of the ethidium bromide–calf thymus DNA (EB–CT-DNA) complex in fluorescence spectra, suggesting that pym-5 could strongly affect the DNA conformation. Furthermore, pym-5 showed the cleavage activity upon the supercoiled plasmid pBR322 DNA in the pBR322 DNA cleavage assay. Our study suggests that DNA may serve as a potential target to these pyrazole derivatives.

Key words pyrazole derivative; synthesis; DNA interaction; molecular docking; spectroscopy
As sequence-specific DNA binding ability has been reported in an increasing number of pyrazole analogs, more attention should be paid to DNA interaction of pyrazole derivatives in the process of drug development.

In our previous study, we synthesized a series of novel pyrazole derivatives designed as kinase inhibitors to be potential antitumor drugs. Their biological activity evaluations elucidated that these compounds exhibited relatively remarkable cell proliferation inhibition and weak multiple kinase activities. Hence, besides the inhibition of kinase leading antitumor activity, another mechanism may exist for the compounds’ inhibition of cell proliferation. Meanwhile, enlightened by the potential DNA-binding ability of pyrazole compounds as mentioned above, we hypothesized that the DNA binding of these 1H-pyrazole-3-carboxamide derivatives may contribute to their antitumor activity. To verify our hypothesis and discover possible off-target effects, four most active compounds from the preliminary screen (Chart 1) were synthesized and tested by DNA-binding experiments to reveal the mechanism of their antitumor activity.

In this paper, we employed a computational method to simulate the binding of these compounds with a known DNA fragment. Considering the energy feasibility on their interactions with DNA predicted by molecular docking, we carried out electronic absorption spectra, fluorescence spectroscopy study, viscosity measurement and DNA cleavage assays to analyze the binding ability and binding mode. Finally, we confirmed that these pyrazole compounds did interact with DNA and the interaction could be an alternative contribution to inhibit cancer cell proliferation by an off-target mechanism.

**Experimental**

**Reagents and Materials** The compounds were synthesized according to the following procedure. Ethidium bromide (EB), calf thymus DNA (CT-DNA) and pBR322 plasmid DNA were purchased from Sigma Chemical Co. Tris–HCl/NaCl (5 mM [Tris(hydroxymethyl)aminomethane] and 50 mM NaCl, and adjusted to pH 7.4 with hydrochloric acid) buffer solution was prepared freshly in triple-distilled water before use. Solvents and other chemicals used were of analytical grade. Solution of the compound and other agents used for strand scission were incubated for an additional 4 h. Culture supernatant was removed and 150 µL of dimethyl sulfoxide (DMSO) (Sangon Biotech, China) was added into each well to make the MTT-formazan crystals completely dissolved. Cell growth inhibition was determined by measuring the absorbance at 570 nm using a microplate reader and calculated according to the equation: Cell growth inhibition = (1–optical density (OD) of treated cells/ OD of control cells) × 100%. The half maximal inhibitory concentrations (IC₅₀) were obtained from liner regression analysis for each tested compound, using GraphPad Prism software.

**Kinase activity** was measured using HTRF assay. On the Beckman Coulter detection platform, HTRF assays were performed in 384-well plates. The following were combined in the reaction mixture: 4 µL of different concentrations of compound (diluted in 100% DMSO at 50 µg/mL), 8 µL of enzyme, and 8 µL of ATP/substrate mix (final concentrations around...
$K_\text{m}$ values). Enzymes, ATP, and substrate were previously diluted in kinase buffer to get final concentrations in the well: 50 mM N-(2-hydroxyethyl)piperazine-N'\textsuperscript{-2}-ethanesulphonic acid (Hepes)/NaOH (pH 7.4), 40 mM NaVO\textsubscript{4}, 0.005% w/v-20, 1 mM dithiothreitol (DTT), and various concentrations of MnCl\textsubscript{2} or MgCl\textsubscript{2}. The ATP/Substrate mixture was added to initiate the assay. The reaction was stopped by addition revelation mixture (55 $\mu$L, ethylenediaminetetraacetic acid (EDTA), 0.33 mM, specific europium-cryptate antibody antiphospho-substrate, and XL-665 with various tags (CisBio International) after being incubated for different times at room temperature. The final concentrations of mixture prepared in revelation buffer in the well: 50 mM Hepes/NaOH (pH 7.4), 0.1% bovine serum albumin (BSA), and 150 mM KF. The reaction was incubated more than 1 h at room temperature, and then detected by Beckman Coulter detector platform. Kinase activities were expressed as a percentage of maximal activity (without compound), resulting from two independent experiments. In all cases, IC\textsubscript{50} values were calculated from replicate curves, using GraphPad Prism software. Staurosporine and Roscovitine were used as positive controls in the kinase assay.

**Molecular Modeling and Docking** Molecular modeling was performed using SYBYL7.3 on a Hewlett-Packard XW4100 PC workstation with a Red Hat Enterprise 4 Linux operating system. All molecules except the DNA were built using SYBYL. The DNA structure was downloaded from the PDB database and prepared by removing both the bound molecules and all waters to avoid potential interference with the docking. Hydrogen was added to the DNA by SYBYL. The compounds were docked into the solution structure of B-DNA dodecamer d(AATCCTTTAAAGATT)\textsubscript{2} (PDB ID:2K4L) with DOCK5.4.\textsuperscript{37}

**Electronic Absorption and Fluorescence Spectra** The DNA-binding experiments were performed at 25°C. Using the electronic absorption spectra, the relative binding of the four complexes to CT-DNA was studied in 5 mM Tris–HCl/50 mM NaCl buffer (pH = 7.4). The compounds were dissolved in a solvent of Tris–HCl buffer (5 mM Tris–HCl, 50 mM NaCl, pH 7.4) at the concentration 1.0×10\textsuperscript{−5} M. Absorption titration experiments were performed with fixed concentration drugs (10 mM) while gradually increasing the concentration of CT-DNA. The ratio of $C_{\text{DNA}}/C_{\text{compound}}$ in each curve decreased as follows: 6/100, 13/100, 25/100, 38/100, 51/100, 76/100, 102/100, 127/100, 152/100, 230/100, 254/100. In order to eliminate the absorbance of CT-DNA itself, an equal amount of CT-DNA was added to both the compound solution and the reference solution during measuring the absorption spectra. By the fluorescence spectral method, the relative binding of compounds to CT-DNA were studied with an EB-bound CT-DNA solution in 5 mM Tris–HCl/50 mM NaCl buffer (pH = 7.4). The ratio of $C_{\text{EB,CT-DNA}}/C_{\text{compound}}$ in each curve decreased as follows: 0/1, 1/0, 1/5, 3/10, 2/5, 1/2, 3/5, 4/5 and 1/1 (Fig. 6). The excitation wavelength was 520 nm for all cases.

**Viscosity Experiments** Viscosity measurements were carried on an Ostwald’s viscometer at 30±0.1°C in a constant temperature bath. The DNA concentration was fixed at 1.5×10\textsuperscript{−4} M and the flow time were measured with a digital stopwatch; the mean values of three measurements were used to evaluate the viscosity of each sample. Data are presented as $(\eta/\eta_0)^{1/3}$ versus $r$ $(r=[\text{compound}]/[\text{DNA}]=0–1)$, where $\eta$ is the viscosity of DNA in the presence of compound and $\eta_0$ is the viscosity of DNA alone. Viscosity values were calculated from the observed flowing time of DNA-containing solutions ($t$) corrected for that of the buffer alone ($t_0$), $\eta=(t/t_0)\eta_0$.

**pBR322 DNA Cleavage Assays** The plasmid DNA cleavage experiments were performed using pBR322 DNA in Tris–HCl buffer. Reactions were performed by incubating DNA (0.05 mM bp) at 37°C in the presence or absence of the compound for the indicated time periods. All reactions were quenched by loading buffer. Agarose gel electrophoresis was carried out on a 1% agarose gel in 0.5× TAE (Tris–acetate–EDTA) buffer containing 0.5 µg/mL EB at 80 V for 1.5 h, then the gel image was captured by the Scion Image System.

**Results and Discussion**

**Synthesis** The synthetic pathways adopted for the preparation of title compounds are illustrated in Chart 2. pym-5, pym-55 and pym-n all bearing a characteristic functional group have a common intermediate (5), which was prepared through alkylation, reduction and acylation. The reduction step of the nitro group to amide employed hydrazine hydrate in the presence of an iron(III) oxide hydroxide (Fe(OH)\textsubscript{3}) as a catalyst\textsuperscript{39–41} in the refluxing 95% ethanol to get a high yield product within 2 h.\textsuperscript{39–41} Furthermore, the catalyst can be recycled after the reaction through washing with anhydrous ethanol. pym-5 bearing a urea group was synthesized from compound 5 with cyclopropylamine employed CDI. pym-55 was prepared using N-hydroxybenzotriazole (HOBt) and 1-ethyl-(3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDCI) as condensing agent to acylate compound 5 by benzoic acid. pym-n was gained through catalyzed hydrogenation with NaBH\textsubscript{4} from the intermediate of Schiff base which was generated by mixing compound 5 with pyridine formaldehyde. The preparation of pym-5 was similar to pym-5 with the starting reactant morphine instead of N-methylpipеразине.

4-Amino-N-[4-[(4-methylpiperazin-1-yl)methyl]phenyl]-1H-pyrazole-3-carboxamide (5): The mixture of N-methyl piperazine (4.9 mL, 44.2 mmol) and triethylamine (12 mL, 86.3 mmol) in dichloromethane (20 mL) was added dropwise to a stirred solution of Nitro-benzyl bromide 1 (10.0 mg, 46.3 mmol) in dichloromethane (100 mL) under the ice-water bath and was refluxed for 1 h. The reaction mixture was extracted with chloroform (100 mL×3), washed with water and saturated sodium chloride each time (100 mL×3). The organic phase was dried with anhydrous magnesium sulfate, filtered, evaporated under vacuum to give pale yellow solid 2. Without further purification, 2 mixed with Fe(OH)\textsubscript{3} (catalyst 2.0 g) and 95% ethanol (100 mL) were kept refluxing and dropwise added the mixture of hydrazine hydrate (25 mL) and 95% ethanol (20 mL), then filtrated when the solution was hot. The residue was washed with hot ethanol (30 mL×2). The solvent was distilled under vacuum to give white solid 3 (6.7 g), then mixed with 4-nitro-1H-pyrazole-3-acid (6.3 g, 40.0 mmol), EDCI (8.4 g, 43.8 mmol) and HOBt (6.0 g, 44.4 mmol) in anhydrous DMF (100 mL), stirred for 24 h at room temperature. The reaction mixture was poured into ice water (200 mL). A large amount of yellow solid precipitation was acquired. The pure product 4 was got from recrystallizing with mixed solvent of methanol and ethyl acetate, then the same process of hydrazine hydrate reduction was done with the catalyst of FeO(OH)/C. The solvent was distilled under vacuum to give
white solid 5 (3.5 g). Yield = 63.9%; mp 199–201°C; IR (KBr) cm⁻¹: 3369, 3227 (NH₂), 1456 (C=O) pyrazole, 1646 (ArH); ¹H-NMR (300 MHz, DMSO-d₆) δ (ppm): 2.1 (3H, s, –CH₃), 2.3–2.5 (8H, m, –CH₂–), 3.3 (2H, s, –CH₂–), 4.7 (1H, t, J = 7.2 Hz, ArH), 7.1–7.2 (2H, m, ArH), 7.7 (2H, d, J = 10.5 Hz, ArH), 9.7 (1H, s, –NHCO–), 12.7 (1H, s, Pyrazole). MS m/z: 315.82 (M⁺); Anal. Calcd for C₁₆H₂₂N₆O: C, 61.13; H, 7.05; N, 26.73; O, 5.09. Found: C, 61.31; H, 6.84; N, 26.52.

5-(3-Cyclopentyldiimide)-N-[4-[(4-methylpiperazinyl)-methyl]phenyl]-1H-pyrazole-3-carboxamide (pym-5): A mixture of 5 (314.0 mg, 1.0 mmol), benzoic acid (112.0 mg, 1.0 mmol), CDI (210.6 mg, 1.3 mmol), EDCI (249.2 mg, 1.3 mmol), HOBT (175.7 mg, 1.3 mmol) were mixed in anhydrous DMF (30 mL), stirred at room temperature. An excess sodium borohydride (500.0 mg) was added, then the reaction mixture was kept refluxing for 2 h and cooled to room temperature. The solution was stirred at room temperature for 3 h, evaporated under vacuum to give pale yellow oily substance. The crude product was separated by column chromatography (developing solvent: methanol–chloroform 1:50) to get pym-5 (200.3 mg). Yield = 48.7%; mp 236–238°C; IR (KBr) cm⁻¹: 3369 (–NH₂) Amie, 3335 (NH), 1446 (C=O), 1377 (C=O) pyrazole, 1593 (ArH), 1656 (O=C–NH), 1174 (C–N); ¹H-NMR (300 MHz, DMSO-d₆) δ: 2.1 (3H, s, –CH₃), 2.4–2.6 (8H, m, –NCH₂–), 4.1 (2H, s, –CH₂–), 6.9–7.0 (3H, m, ArH), 7.1 (2H, d, J = 8.7 Hz, ArH), 7.2 (1H, m, ArH), 7.7 (2H, d, J = 8.7 Hz, ArH), 8.0 (1H, s, ArH), 8.2 (1H, m, ArH), 8.9 (1H, s, –NHCO–), 10.0 (1H, s, –NHCO–), 13.2 (1H, s, pyrazole). MS m/z: 419.30 (M⁺); Anal. Calcd for C₁₆H₂₈N₇O₂: C, 66.01; H, 6.29; N, 20.08; O, 7.65. Found: C, 65.98; H, 6.43; N, 20.35.

N-[4-[(4-Methylpiperazin-1-yl)methyl]phenyl]-5-(pyridin-4-yl-methyl)-1H-pyrazole-3-carboxamide (pym-n): The mixture of 5 (314.0 mg, 1.0 mmol), pyridine formaldehyde (0.5 mL) and methanol (30 mL) was heated to reflux, then cooled to room temperature. An excess sodium borohydride (500.0 mg) was added, then the reaction mixture was kept refluxing for 2 h and evaporated under vacuum to give pale yellow oily substance and separated by column chromatography (developing solvent: methanol–chloroform 1:20) to get pym-5 (222.0 mg). Yield = 56.1%; mp 156–158°C; IR (KBr) cm⁻¹: 2995 (CH₂), cyclopropyl, 1646 (C–NH), 1593 (ArH), 1666 (O=C–NH), 1130 (C–N); ¹H-NMR (300 MHz, DMSO-d₆) δ: 0.4–0.6 (4H, m, –CH₂–), 2.1 (3H, s, –CH₃), 2.3–2.7 (9H, m, –CH₂–, –CH–), 3.3 (2H, s, –CH₂–), 7.2 (2H, d, J = 8.4 Hz, ArH), 7.3 (1H, s, ArH), 7.7 (2H, d, J = 8.4 Hz, ArH), 8.0 (1H, s, –NHCONH–), 8.78 (1H, s, –NHCO–), 10.0 (1H, s, –NHCO–), 13.1 (1H, s, pyrazole). MS m/z: 396.94 (M⁺); Anal. Calcd for C₂₀H₂₇N₇O₂: C, 65.02; H, 6.85; N, 24.45. Found: C, 60.32; H, 7.06; N, 24.45.

5-(3-Cyclopentyldiimide)-N-[(morpholinomethyl)phenyl]-1H-pyrazole-3-carboxamide (pyz-5): The preparation process of pym-5 which began with morphine was the same as pym-5. Yield = 56.1%; mp 121–123°C; IR (KBr) cm⁻¹: 3335 (NH), 1446 (C=O), 1454 (C–N) pyrazole, 2995 (CH₂) cycloproyl, 1666 (O=C–NH), 1533, 1265 (amide), 1066 (C=O) ether;
sugar residues compared to others, and meanwhile, the potential electronegative phosphorous group. In addition, the pyrazole formed a smaller steric-negative interaction with DNA, so binding affinity with the lowest binding energy. Compared mol, respectively. Among them, had the strongest favorable than the morpholine of pH, the protonation of N atom on the piperazine ring is more exhibited a higher binding affinity. Also, at physiological values for CDK2, EGFR and ABL2 of four compounds (HepG2) respectively. However, the targeted kinase inhibition values of pym-5 and pym-n were not comparable to the positive drug.

### In Vitro Pharmacological Evaluation
The antiproliferative activity of the four selected compounds was evaluated by MTT tetrazolium dye assays on HCT116 cells and HepG2 cells (Table 1). Among the four compounds, the inhibition IC_{50} values of pym-5 and pym-55 are similar to the positive drug Iressa (IC_{50}=9.34\,\mu\text{M}, HCT116) and Roscovtine (IC_{50}=9.87\,\mu\text{M}, HepG2) respectively. However, the targeted kinase inhibition IC_{50} values for CDK2, EGFR and ABL2 of four compounds are similar, and the detailed view of the DNA binding ability of four pyrazole compounds (pym-5, pyz-5, pym-55 and pym-n) to calf thymus DNA (CT-DNA) was studied by electronic absorption spectroscopy. The UV-Vis spectra were measured in the way: Fixing the concentration of compounds (C_{compound}=3.00\times10^{-5}\text{m}) while increasing the concentration of CT-DNA. The tendency of hypochromism without obvious red shift occurred in four compounds can be observed in Fig. 2, which suggests that the interaction of compounds to DNA is not from a classic intercalation mechanism but probably groove binding mode. The absorption relationship between the compounds and DNA can be expressed by double reciprocal equation:

\[ \frac{1}{A} = \frac{1}{A_0} + \frac{1}{K_a \times A_0 \times C_{DNA}} \]  

(1)

\[ \Delta A = A - A_0 \]  

(2)

\[ 1/\Delta A = -1/\left( K_a \times A_0 \times C_{DNA} \right) - 1/A_0 \]  

(3)

Where \( A_0 \) and \( A \) are the absorbance of compounds without and in the presences of DNA, respectively. \( K_a \) is the binding affinity.

### Study of the Interaction with DNA. Molecular Modeling of Compound–DNA Interactions
Molecular docking was applied to investigate the molecular mechanism of DNA-ligand binding. The four compounds all interact with the base pairs of DNA via groove binding mode. Docking modes of the four compounds are similar, and the detailed view of the DNA binding mode of pym-5 is shown in Fig. 1. The molecule is shaped as a chain by two amide bonds on the pyrazole ring, which enables the molecule to properly stretch along the DNA phosphate-deoxyribose backbone. Due to the factors stated above, pym-5 would penetrate deeply into the minor groove of DNA and exhibit a better DNA binding affinity.

Electronic Absorption Spectral Studies
The potential binding ability of four pyrazole compounds (pym-5, pyz-5, pym-55 and pym-n) to calf thymus DNA (CT-DNA) was studied by electronic absorption spectroscopy. The UV-Vis spectra were measured in the way: Fixing the concentration of compounds (C_{compound}=3.00\times10^{-5}\text{m}) while increasing the concentration of CT-DNA. The tendency of hypochromism without obvious red shift occurred in four compounds can be observed in Fig. 2, which suggests that the interaction of compounds to DNA is not from a classic intercalation mechanism but probably groove binding mode.

Table 1. Antiproliferative Activity and Kinase Inhibition of Four Derivatives

| Compd. | Cell line inhibition IC_{50} (\mu\text{M}) | Kinase inhibition IC_{50} (\mu\text{M}) |
|--------|------------------------------------------|----------------------------------------|
|        | HepG2 | HCT116 | CDK2 | EGFR | ABL2 |
| pym-5  | 32.23 | 15.94  | 7.23 | 3.31 | 2.05 |
| pym-n  | 89.5  | 10.93  | 9.5  | 13.51| 3.98 |
| pym-55 | 15.96 | NT     | 10.96| 32.67| 12.24|
| pyz-5  | 244.18| NT     | 6.18 | 7.92 | 2.34 |
| Iressa | NT    | 9.34   | NT   | NT   | NT   |
| Roscovtine | 9.87 | NT     | 0.01 | NT   | NT   |
| Staurosporine | NT | NT     | 0.11 | 0.054|     |

\[ ^1\text{H-NMR (300 MHz, DMSO-}\text{d}_6) \delta: 0.7-1.0 (4H, m, –CH_2–), 2.5 (4H, m, –NCH_2–), 2.7 (1H, m, –CH–), 3.3 (2H, s, –CH_2–), 3.6 (4H, m, –OCH_2–), 7.0 (1H, m, AR-H), 7.2 (2H, d, J=8.7Hz, AR-H), 7.7 (2H, d, J=8.7Hz, AR-H), 10.0 (1H, s, –NHCO–), 11.3 (1H, s, –NHCONH–), 12.0 (1H, s, –NHCONH–), 14.0 (1H, s, pyrazole); MS m/z: 383.90 (M^+); Anal. Calcld for C_{19}H_{24}N_{6}O_{3}: C, 59.36; H, 6.29; N, 21.86; O, 12.49%. Found: C, 59.65; H, 6.63; N, 21.55. \]

\[ \text{IC}_{50}^\text{a} \text{ values are the mean from two independent dose–response curves; variation was generally (±25%) with the reference compounds with Iressa and Roscovtine. NT, not tested.} \]
constant between the compounds and DNA, and $C_{DNA}$ is the concentration of DNA. In the plot of $\Delta A$ versus $1/C_{DNA}$ (Fig. 3), $K_b$ is then given by the ratio of the intercept to slope intercept listed in Table 2, which indicates that the docking result of the DNA binding order is in accordance with the result evaluated by electronic spectra that is $pym-5 > pyz-5 > pym-55 > pym-n$.

**Fluorescence Spectroscopic Studies** With higher sensitivity and accuracy, fluorescence spectroscopic study was carried out to further clarify the interaction mode of four
compounds with DNA. No luminescence was observed at room temperature in the aqueous solution, neither in any organic solvent examined. Thus, the experiment was carried out in the EB background. EB molecule can make a strong interaction with the adjacent DNA base pairs and emit intense fluorescence at about 550–650 nm in the hydrophobic environment provided by the groove of DNA. This fluorescence would be quenched when the compound squeezed out of the EB molecule from the hydrophobic region and exposed the EB molecule to the polarity micro-environment by inserting into the groove or the internal structure of the DNA. Therefore, the quenching degree can be used to measure the binding propensity of the compound to DNA. As the concentration of the compound was increased, all the compounds showed distinctive decrease happened to the intensity of EB–CT-DNA system. The most interesting spectrum was pym-5, in Fig. 4, more than 50% decrease of the emission intensity of the EB–CT-DNA (1×10^{-4} M) complex with the compound pym-5 (3×10^{-5} M) being added, which indicates a strong interaction between pym-5 and DNA. Based on the classical theory of fluorescence quenching: 

\begin{equation}
F/F_0 = 1 + K_q [Q]
\end{equation}

The plot of \( F/F_0 \) versus \([Q]\) (\([Q]\) is the concentration of quencher compound) is linear for either simple dynamic quenching or static quenching. Hence, a broken line shown in Fig. 5a reveals that two dominant quenching processes exist in the system possibly. The turning point was found at the concentration of 4×10^{-5} M. That means the mechanism of the quenching process changed at this concentration. \( K_q \) (1.8×10^{12} M^{-1}s^{-1}) given by the ratio of the slope to intercept in the second stage of this plot is greater than other kinds of quencher (the maximum diffusion of biological macromolecules sudden collision off constant \( K_q \) is 2×10^{10} M^{-1}s^{-1}), suggesting that the second quenching is a static quenching process. The binding constant \( K \) and the site number of n were calculated by Eq. (5), \( K \) and \( n \) were 8.57×10^{5} M^{-1} and 1.38, respectively shown in Fig. 5b.

\begin{equation}
\log([F_0 - F]/F) = \log + n \log([Q])
\end{equation}

### Viscosity Measurement

Spectra probes generally provide necessary, but not more persuasive evidence than viscosity measurement. The value of viscosity is sensitive to the length change of DNA. The distance between adjacent base pair will be larger, when the molecule intercalates into DNA, causing obviously lengthening. In other way of interaction model (electrostatic attraction or groove binding), due to no increase in the DNA length the viscosity of the sample solu-

### Table 2. The Docking Results and DNA-Binding Constants

| Compound | pym-5 | pyz-58 | pym-55 | pym-n |
|----------|-------|--------|--------|--------|
| Binding energy (kcal/mol) | -49.67 | -44.56 | -30.91 | -28.80 |
| Binding constant K (L/mol) | 1.06×10^4 | 8.83×10^4 | 6.85×10^4 | 1.64×10^4 |

Fig. 4. Representative Fluorescence Emission Spectra of DNA-EB in Tris–HCl Buffer (pH 7.4) with the Increasing in Molar Ratio of pym-5 to DNA (0, 1/10, 1/5, 3/10, 2/5, 1/2, 3/5, 4/5, 1/1) at 25°C

Fig. 5. (a) Stern–Volmer Plots for the Fluorescence Quenching of the EB–CT-DNA Complex with the Compound pym-5 and (b) \( \log([F_0 - F]/F) \) of \( \log([Q]) \) for the Linear
Control; Lane 2: (1 × 0.45, 0.6, 0.8, 1) in Fig. 7a. 49) A control sample dependent manner for pym-5 version of Form I to Form II is observed in a concentration-pounds performed no cleavage effect in the assay. The con - pyme-5 cleavage promoted by (Form II) that we can find in Fig. 7 (pBR322 DNA hydrolytic the Form I relax to generate a slower-moving nicked form when circular pBR322 DNA is subjected to electrophoresis, the age reaction can be monitored by agarose gel electrophoresis. The cleav - employed to incubate with pBR322 DNA under the reaction conditions to assess the chemical nuclear activity. The cleav - tion changes litter. In Fig. 6, the minor change in viscosity for 322 Plasmid DNA (0.25 µg/L) with the Addition of Four Compounds Separately in Different Concentration under Dark for 1.5h, the Result Shown in a (pym-5), b (pym-55), c (pym-n), d (pyz-5) and Lane 1: DNA Control; Lane 2: (1×10⁻¹⁰ M); Lane 3: (5 × 10⁻¹⁰ M); Lane 4: (5×10⁻⁸ M); Lane 5: (5×10⁻⁹ M); Lane 6: pym-5 (5×10⁻⁸ M); Lane 7: (5×10⁻⁶ M); Lane 8: (5×10⁻³ M); Lane 9: (5×10⁻⁷ M); Lane 10: (5×10⁻³ M)

Fig. 7. Agarose Gel Electrophoresis Patterns for the Cleavage of pBR 322 Plasmid DNA (0.25 µg/µL) with the Addition of Four Compounds Separately in Different Concentration under Dark for 1.5h, the Result Shown in a (pym-5), b (pym-55), c (pym-n), d (pyz-5) and Lane 1: DNA Control; Lane 2: (1×10⁻¹⁰ M); Lane 3: (5 × 10⁻¹⁰ M); Lane 4: (5×10⁻⁸ M); Lane 5: (5×10⁻⁹ M); Lane 6: pym-5 (5×10⁻⁸ M); Lane 7: (5×10⁻⁶ M); Lane 8: (5×10⁻³ M); Lane 9: (5×10⁻⁷ M); Lane 10: (5×10⁻³ M)


cleavage activity of pym-5 may be attributed to a hydrolysis of pBR322DNA based on the nucleophile attacking phospho - nates by the uramido group. Also, cationic protonated tertiary amine under the pH7.4 may stabilize the negatively charged nuclear phosphorus penta-coordinated transition state. 50)

Conclusion
In this study, four compounds with the skeleton of 1H-pyr - azole-3-carboxamide were synthesized in moderate reaction condition and high yield. In the in vitro pharmacological evaluation, they exhibited significant inhibition against two cancer cell lines (HCT116, HepG2) and weak kinase inhibition. Then molecular docking combined with spectra experiments and viscosity measurements were employed to investigate their potential DNA-damaging ability and DNA interaction mode. The concentration dependence of hypochromism in the electronic absorption spectra demonstrated the validity and reliability of mode predicted by the docking. Meanwhile, the result of binding constants calculated from the UV absorption experiment was consistent with the rank order of binding energy obtained from docking. Besides, the efficient competitive binding ability in fluorescence spectra and chemical nuclease activity for DNA strand scission in Agarose gel electrophoresis were observed in pym-5. In summary, compound pym-5 was identified to have a significant off-target interaction with DNA, which may contribute to inhibiting the proliferation of cancer cells by DNA damage mechanism. Furthermore, the study emphasized the possible off-target effects based on the specific characteristics of the pyrazole group in the kinase inhibitor discovery.

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References and Notes
1) Haggins D. J., Sherman W., Tidor B., J. Med. Chem., 55, 1424–1444 (2012).
2) Bible K. C., Bible R. H. Jr., Kottke T. J., Svingen P. A., Xu K., Pang Y.-P., Hajdu E., Kaufmann S. H., Cancer Res., 60, 2419–2428 (2000).
3) Zhai S., Senderowicz A. M., Sausville E. A., Figg W. D., Ann. Pharmacother., 36, 905–911 (2002).
4) Baraldi P. G., Bovero A., Fruttarolo F., Preti D., Tabrizi M. A., Pa - viani M. G., Romagnoli R., Med. Res. Rev. 24, 475–529 (2004).
5) Cozzi P., Farmaco, 56, 57–65 (2001).
6) Dervan P. B., Doss R. M., Marques M. A., Curr. Med. Chem. Anti - cancer Agents, 5, 373–387 (2005).
7) Baraldi P., del Carmen Nunez M., Espinosa A., Romagnoli R., Curr. Top. Med. Chem., 4, 231–239 (2004).
8) S. Goodsell D., Curr. Med. Chem., 8, 509–516 (2001).
9) Pal D., Saha S., Singh S., Int. J. Pharm. Pharm. Sci., 4, 98–104 (2012).
10) Tambe S. K., Dighe N. S., Pattan S. R., Kedar M. S., Musmade D. S., Pharmacologyonline, 2, 5–16 (2010).
11) Bekhit A., Ashour H., Bekhit A., Bekhit S., Med. Chem., 5, 103–117
(2009).
12) Fusteró S., Sánchez-Roselló M., Barrio P., Simón-Fuentes A., Chem. Rev., 111, 6984–7034 (2011).
13) Wang L., Yu X., Feng X., Bao M., J. Org. Chem., 78, 1693–1698 (2013).
14) Willy B., Müller T. J. J., Org. Lett., 13, 2082–2085 (2011).
15) Tran G., Gomez Pardo D., Tsuchiya T., Hillebrand S., Vors J.-P., Cossy J., Org. Lett., 15, 5550–5553 (2013).
16) Savant M. M., Pansuriya A. M., Bhuva C. V., Kapuriya N., Patel A., J. Org. Chem., 83, 206–212 (2010).
17) Xie L., Qian X., Cui J., Xiao Y., Wang K., Wu P., Cong L., Bioorg. Med. Chem., 16, 8713–8718 (2008).
18) Poli M., Prasad V. S. P., Mayur Y. C., J. Comb. Chem., 12, 176–180 (2010).
19) Baraldi P. G., Balzani V., Pavan G. M., Spalluto G., Tabrizi M. A., R. Med. Chem., 51, 3124–3132 (2008).
20) Molinari A., Oliva A., Ojeda C., del Corral J. M. M., Castro M. A., Cuevas C., San Feliciano A., Arch. Pharm. (Weinheim), 342, 591–599 (2009).
21) Sakui K., Tomita Y., Ue T., Goshima K., Ohminato M., Tsubomura T., Matsumoto K., Ohmura K., Kawakami K., Inorganica Chim Acta, 297, 64–71 (2000).
22) Cao L., Qian H., Wang Y., Wang J. W., Acta Crystallogr. Sect. E Struct. Rep. Online, 65, o1158 (2009).
23) Zhan Z.-Y., Dervan P. B., J. Med. Chem., 51, 3124–3132 (2008).
24) Liu C.-S., Zhang H., Chen R., Shi X.-S., Bu X.-H., Yang M., Acta Crystallogr. Sect. E Struct. Rep. Online, 68, o2513 (2012).
25) Gozalbes R., Simon L., Florell N., Sartori E., Monteils C., Baudelle R., J. Med. Chem., 51, 3124–3132 (2008).
26) Yin L., Wang Y., Wang Y. Y., Wang J. W., Acta Crystallogr. Sect. E, 59, 65–66 (2002).
27) Baraldi P. G., Beria L., Cozzi P., Gerini C., Espinosa A., Gallo M. A., Entrena A., Bingham J. P., Hartley J. A., Romagnoli R., J. Med. Chem., 44, 2536–2543 (2001).
28) Perczel E. D., Balzarini J., Bando T., Sugiyama H., Romagnoli R., J. Photochem. Photobiol. B, 97, 521–525 (2010).
29) Prachnik F. F., Jakimowicz P., Czuni Z., Zakrzewska-Czerwinska J., Opolaski A., Wietrzyk J., Inorganica Chim Acta, 297, 64–71 (2000).
30) Liu C.-S., Zhang H., Chen R., Shi X.-S., Bu X.-H., Yang M., Chem. Pharm. Bull., 55, 996–1001 (2007).
31) Purohit M., Prasad V. S. P., Mayur Y. C., Arch. Pharm. (Weinheim), 344, 248–254 (2011).
32) Yao J. Y., Xiao J., Zhao H., Acta Crystallogr. Sect. E Struct. Rep. Online, 65, o1158 (2009).
33) Zhan Z.-Y., Dervan P. B., Bioorg. Med. Chem., 8, 2467–2474 (2000).
34) Xie L., Qian X., Cui J., Xiao Y., Wang K., Wu P., Cong L., Bioorg. Med. Chem., 16, 8713–8718 (2008).
35) Roos R., Bloch I., Sklenar H., Shakked Z., Nucleic Acids Res., 33, 7048–7057 (2005).
36) Sun J., Huang Y. R., Harrington W. R., Sheng S., Katzenellenbogen J. A., Katzenellenbogen B. S., Endocrinology, 143, 941–947 (2002).
37) Antinon D., Barata T., Jenkins T. C., Parkinson G. N., Howard P. W., Thurston D. E., Zloh M., Biochemistry, 47, 11818–11829 (2008).
38) Experimental procedures for the synthesis catalyst: In a 250mL single-necked, round-bottomed flask equipped with a magnetic stirrer, activated carbon (5 g) was added into a solution of FeCl₃·6H₂O (0.7g, 2.6mmol) in 100mL distilled water. Then we added the NaOH (0.4g, 10mmol) in to the mixture in batches. The temperature of the brown mixture was raised slowly to 60°C in 2h and kept the temperature for 6h. After centrifugation and drying, the catalyst was milled to a fine powder.
39) Benz M., van der Kraan A. M., Prins R., Appl. Catal. A Gen., 172, 149–157 (1999).
40) Benz M., Prins R., Appl. Catal. A Gen., 183, 325–333 (1999).
41) Lauwiner M., Rys P., Wissmann J., Appl. Catal. A Gen., 172, 141–148 (1999).
42) Saito S. T., Silva G., Pungartnik C., Brendel M., J. Photochem. Photobiol. B, 111, 59–63 (2012).
43) Arshad N., Abbas N., Bhatti M. H., Rashid N., Tahir M. N., Saleem S., Mirza B., J. Photochem. Photobiol. B, 117, 228–239 (2012).
44) Zhang S., Ling B., Qu F., Sun X., Spectrochim. Acta A Mol. Biomol. Spectrosc., 97, 521–525 (2012).
45) Zhang G., Hu X., Zhao N., Li W., He L., Pestic. Biochem. Physiol., 98, 206–212 (2010).
46) Mansouri M., Pirouzi M., Saberi M., Ghaderabad M., Chamani J., Molecules, 18, 789–813 (2013).
47) Ray A., Seth B. K., Pal U., Basu S., Spectrochim. Acta A Mol. Biomol. Spectrosc., 92, 164–174 (2012).
48) Chi Z., Liu R., Sun Y., Wang M., Zhang P., Gao C., J. Hazard. Mater., 175, 274–278 (2010).
49) Selvakumar B., Rajendiriran V., Uma Maheswari P., Stoeckli-Evans H., Palaniandavar M., J. Inorg. Biochem., 106, 316–330 (2006).
50) Williams N. H., “DNA Hydrolysis: Mechanism and Reactivity, in Artificial Nucleases,” Vol. 13, Springer, Berlin, Heidelberg, 2004.