Full Paper

Some Pyrazole and Pyrazolo[3,4-*d*]pyrimidine Derivatives: Synthesis and Anticancer Evaluation

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5-Amino-1-*p*-tolyl-1*H*-pyrazole-4-carbonitrile (**1**) was used for the preparation of some novel pyrazoles and pyrazolo[3,4-*d*]pyrimidines **2**–**10**. Moreover, the cytotoxicity and *in vitro* anticancer activities of the prepared compounds were also assessed against the MCF-7 breast cancer, HepG2 liver cancer, and A549 lung carcinoma cell lines, along with investigation of the effect of the synthesized compounds on the expression of urokinase plasminogen activator (uPA). The tested compounds exhibited remarkable cytotoxic activity against MCF-7 and HepG2 cells. Among the tested compounds, **2** and **9** revealed promising anticancer activity compared to the activity of the commonly used anticancer drug, doxorubicin, by inhibiting the expression of uPA.

Keywords: Anticancer / Pyrazole / Pyrazolopyrimidine / Pyrazolothiazolopyrimidine / Urokinase

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Introduction

Targeted therapies have a high specificity toward tumor cells, providing a broader therapeutic window with less toxicity. They are also often useful in combination with cytotoxic chemotherapy or radiation to produce additive or synergistic anticancer activity because their toxicity profiles often do not overlap with traditional cytotoxic chemotherapy. Thus, targeted therapies represent a new and promising approach to cancer therapy, one that is already leading to beneficial clinical effects. There are multiple types of targeted therapies available, including monoclonal antibodies, inhibitors of tyrosine kinases, and antisense inhibitors of growth factor receptors [1].

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Urokinase plasminogen activator (uPA) is a serine protease that functions in the conversion of the circulating plasminogen to the active, broad-spectrum serine protease, plasmin. uPA is secreted as an inactive single-chain proenzyme by many different cell types and exists in a soluble or cell-associated form by binding to a specific membrane uPA receptor (uPAR) [2, 3]. The uPA is involved in many physiological functions and, along with members of the matrix metalloproteinases (MMPs) family, it has been implicated in cancer invasion and metastasization [4–6]. Besides the proteolytic function, upon binding to uPAR, uPA is involved in initiating versatile intracellular signal pathways that regulate cell proliferation, adhesion, and migration through its interaction with various integrins and vitronectin [7]. Urokinase is implicated in a large number of malignancies, e.g. cancers of breast, lung, bladder, cervix, kidney, stomach, and brain [8, 9]. Also, the expression of urokinase is associated with tumor growth and invasion and may be a useful prognostic factor for hepatocellular

carcinoma [10]. The role of uPA in human cancer progression is further supported by clinical evidences demonstrating that high tissue levels of its components correlate with a poor prognosis in different types of cancer such as breast and gastrointestinal cancers [11].

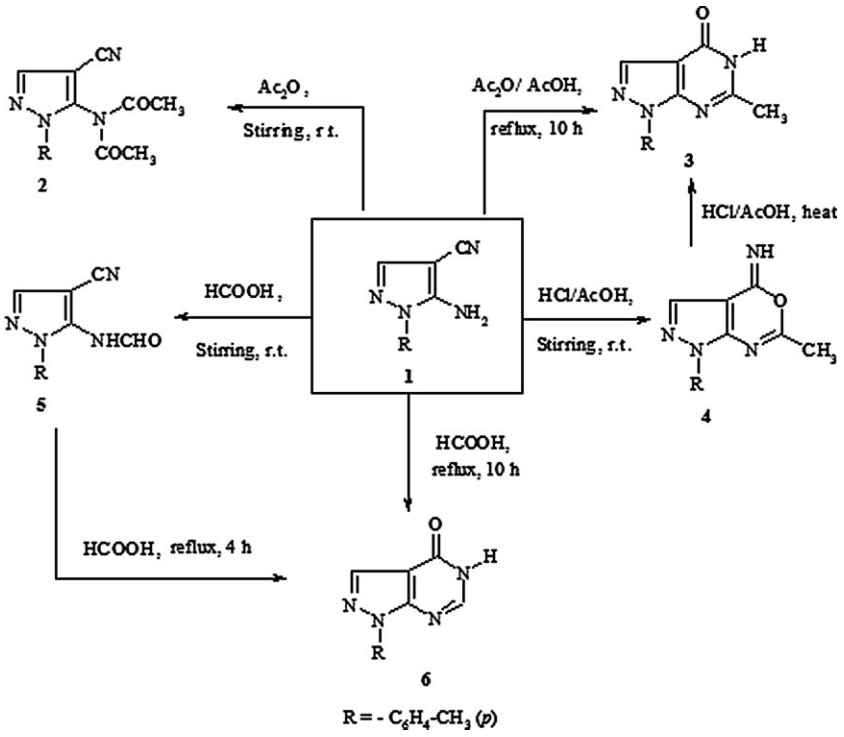
On the other hand, the chemistry of pyrazole ring system has attracted much attention as it shows diverse properties [12–14]. These compounds are not only important from a viewpoint of biological activities as potential antimicrobial [15, 16], anti-inflammatory [17], antiviral [18], and anticancer agents [19], but also useful as starting compounds for preparation of other fused pyrazolopyrimidine derivatives of considerable chemical and biological importance as purine analogs [20, 21]. In addition, it was reported that several pyrazolopyrimidines have xanthine oxidase inhibitory activity [22–24], and showed significant antimicrobial [25, 26], antitumor [27], and antiviral activity [28, 29]. For small organic molecules, simple nitrogen-containing heterocycles receive a large amount of attention in the literature; of these heterocycles, the synthesis, reactions, and biological activities of azole- and azoloazines-containing molecules stand as an ever expanding area of research in hetero aromatic chemistry [30–32].

In previous reports [33–37], replacement of the nitrogen proton in position-1 of the pyrazole ring in pyrazolopyrimidine derivatives by some other substituents drastically enhancees its biological activities. Keeping this in mind, the aim of this work is to prepare some novel pyrazole and pyrazolopyrimidine derivatives bearing a *p*-tolyl moiety for the purpose of developing their biological effect as anticancer agents.

Results and discussion

Chemistry

5-Amino-1-*p*-tolyl-1*H*-pyrazole-4-carbonitrile (**1**) [38] was used as the key compound for this study. Thus, when compound **1** was stirred at room temperature with acetic anhydride, it gave the *N*-acetyl-2*H*-pyrazol-3-yl-acetamide derivative **2** (Scheme 1). The structure of the latter compound was confirmed on the basis of its spectral data (cf. Experimental section, Scheme 1). The IR spectrum of compound **2** showed absorption bands assignable to the C≡N and two C=O groups. While carrying out the same reaction under reflux in the presence of glacial acetic acid, annulations took place to give 6-methyl-1-*p*-tolyl-1,5-dihydro-pyrazolo[3,4-*d*]pyrimidin-4-one (**3**). The absence of absorption band characteristic for C≡N group and the presence of C=O in the IR and ^{13}C NMR spectra of compound **3** confirmed its structure. Also, the ^{1}H NMR spectrum of the pyrimidinone derivative **3** showed signals at 9.42 and 2.64 ppm for NH and C_6-CH_3, respectively (cf. Experimental section).

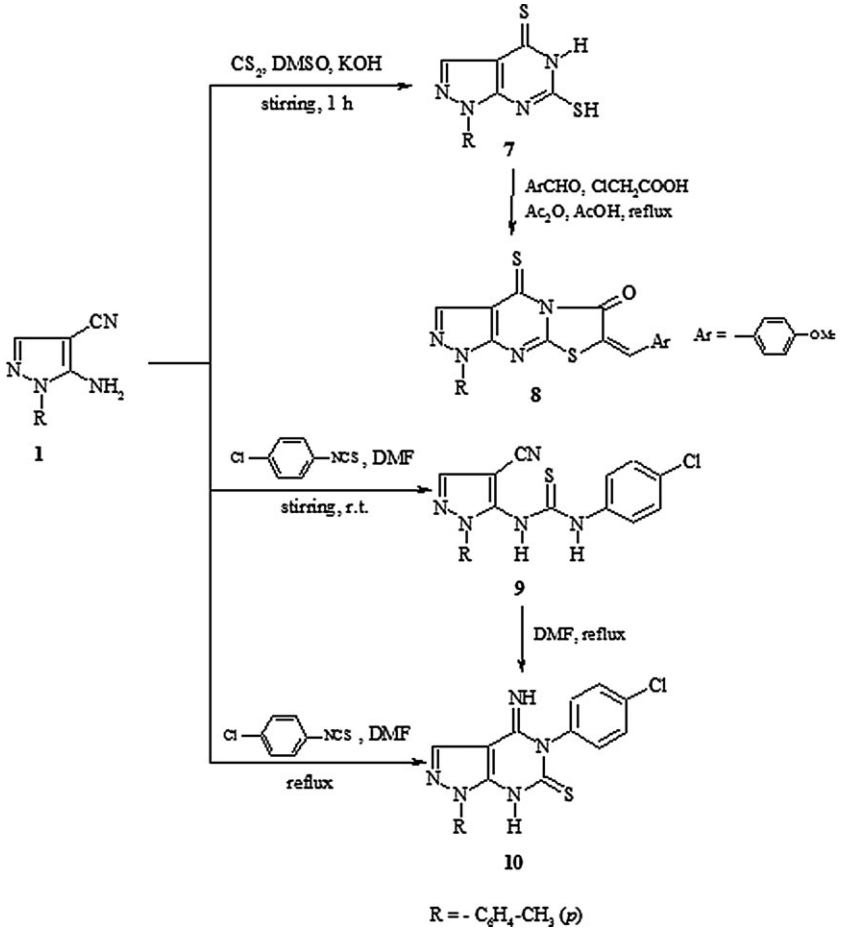


Scheme 1. Synthesis of compounds **2–6**.

However, treatment of compound **1** with a mixture of HCl/AcOH at room temperature gave the [1,3]oxazine derivative **4** [39]. The IR spectrum of the latter compound showed the absence of absorption band characteristic for C≡N group and the presence of imine NH at 3210 cm^{-1}, and the ^{1}H NMR spectrum showed signals at 12.10 and 2.32 ppm for =NH and C_6-CH_3, respectively (cf. Experimental section). Moreover, heating of the oxazine derivative **4** in a mixture of HCl/AcOH for 2 h gave compound identical in all respects to derivative **3**.

Meanwhile, treatment of the 5-amino-1-*p*-tolyl-1*H*-pyrazole-4-carbonitrile (**1**) with formic acid, at room temperature, gave the *N*-formyl derivative **5** (Scheme 1). The IR and ^{13}C NMR spectra of compound **5** showed absorption bands for C≡N and HC=O groups. While, pyrazolo[3,4-*d*]pyrimidin-4-one **6** was obtained either by heating of compound **1** or compound **5** with formic acid. The absence of absorption band characteristic for C≡N group and the presence of C=O group in the IR and ^{13}C NMR spectra of compound **6** confirmed its structure. In addition, the ^{1}H NMR spectrum of the pyrimidinone derivative **6** showed signals at (δ, ppm) 10.10 and 8.55 ppm for NH and C_6-H, respectively (cf. Experimental section).

On the other hand, treatment of compound **1** with carbon disulfide afforded the thione derivative **7**. The ^{1}H NMR spectrum of the latter compound showed the presence of the SH and NH groups at 4.50 and 11.30 ppm (exchangeable with D_2O) in addition to the peak corresponding to the C=S group at 170.23 ppm in its ^{13}C NMR spectrum (cf. Experimental section). Compound **7** was treated with chloroacetic acid and 4-methoxybenzaldehyde in glacial acetic acid/acetic anhydride in the presence of sodium acetate to give the pyrazolothiazolopyrimidine derivative **8** (Scheme 2). Moreover, on stirring of compound **1**, in dimethyl formamide, with *p*-chlorophenyl isothiocyanate at room temperature afforded the corresponding thiourea derivative **9**. The MS spectrum of the latter compound gave fragments of the isotopic pattern due to the presence of chlorine atom (cf. Experimental section). Moreover, on heating of compound **9** in dry dimethyl formamide, it cyclized to pyrazolo[3,4-*d*]pyrimidine-6(5*H*)-thione derivative **10**. The latter compound was also obtained



Scheme 2. Synthesis of compounds **7–10**.

by refluxing compound **1,** dissolved in dimethyl formamide, with 4-chlorophenyl isothiocyanate for 6 h. The IR spectrum of compound **10** revealed the absence of the C≡N group as well as the presence of fragments showing the isotopic pattern due to the presence of chlorine atom in MS spectrum (cf. Experimental section).

Anticancer evaluation

In vitro cytotoxic activity

The cytotoxicity of the tested compounds **1–10** was tested using SRB assay as described by Skehan et al. [40] in breast cancer cell line MCF-7, liver cancer cell line HepG2, and lung carcinoma cell line A549. For comparison, doxorubicin was also tested and the results revealed that all the compounds did not exert any activity against lung carcinoma cell line A549. In case of breast cancer cell line MCF-7 and liver cancer cell line HepG2 compound **2** (IC_{50}: 2.60 ± 0.27 and 3.75 ± 0.44 μg/mL, respectively) exhibited similar activity to doxorubicin (IC_{50}: 2.80 ± 0.24 and 3.75 ± 0.35μg/mL, respectively). The order of cytotoxic activity of the tested compounds was **2, 9, 5, 3, 1, 6, 4, 10, 7**, and **8** in a descending order.

The level of uPA protein expression

To identify the mechanism of action responsible for the cytotoxicity of tested compounds **1–10**, the level of uPA protein expressed in the two cell lines (breast cancer cell line MCF-7 and liver cancer cell line HepG2) was estimated quantitatively. The result revealed that the data of uPA expression were inconsistent with the cytotoxic activity. In case of breast cancer cell line MCF-7 and liver cancer cell line HepG2, the level of uPA decreased in compounds by the following percent in MCF-7 and HepG2, respectively: **1** (66, 60%), **2** (90, 87%), **3** (77, 63%), **4** (54, 48%), **5 (**82, 67%), **6** (60, 56%), **7** (7, 5%)**, 8 (**3, 5%), **9 (**86, 83%), and **10** (9, 6%). From the results, compound **2** exhibited a good activity in MCF-7 (90%) and HepG2 cells (87%) similar to doxorubicin (91 and 88%, respectively) (Table 1). In both MCF-7 and HepG2 cells, the order of uPA activity inhibition of the tested compounds was **2, 9, 5, 3, 1, 6, 4, 10, 7,** and **8** in a descending order, which is in accordance with the cytotoxic activity.

Taken together, these findings suggested that there is correlation between the cytotoxicity of the tested compounds and inhibition of the urokinase activity. The tested compounds exert anti-carcinogenic activity in MCF-7 breast and HepG2 liver cancer cells by inhibiting the activity of urokinase enzyme, which may reduce the cell proliferation and result in significant growth inhibition.

Conclusion

In conclusion, the present results suggested that there are correlations between the cytotoxic activity of the tested compounds **1–10** and inhibition of the urokinase activity. The tested compounds exert anti-carcinogenic activity in hepatic HepG2 and breast MCF-7 cancer cell lines through down-regulation of the activity of urokinase enzyme, which may reduce cell proliferation and result in significant growth inhibition, especially compounds **2** and **9** that revealed promising activity compared to the activity of the commonly used anticancer drug, doxorubicin. The structure–activity relationship (SAR) of the tested compounds established that the pyrazole derivative **1** (starting material) revealed moderate activity, whereas substituted amino group as in derivatives **2**, **5**, and **9** increased the activity. However, fusing another ring (oxazine, pyrimidine, or fused pyrimidine) to the

Table 1. *In vitro* cytotoxic activity and the percent inhibition of uPA of the synthesized compounds on different cell lines.

	IC_{50} (μg/mL)			% inhibition of uPA[a)]		
Compound	**MCF-7**	**HepG2**	**A549**	**MCF-7**	**HepG2**	**A549**
Doxorubicin	2.80 ± 0.24	3.90 ± 0.37	4.11 ± 0.40	91 ± 3.70	88 ± 6.36	82 ± 6.35
DMSO	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
1	5.90 ± 0.56	8.00 ± 0.92	N.A.	66 ± 5.44	60 ± 5.09	N.A.
2	2.60 ± 0.27	3.75 ± 0.44	N.A.	90 ± 5.76	87 ± 6.45	N.A.
3	5.00 ± 0.63	6.20 ± 0.80	N.A.	77 ± 4.28	63 ± 4.65	N.A.
4	6.30 ± 0.60	7.25 ± 0.35	N.A.	54 ± 4.93	48 ± 4.82	N.A.
5	4.60 ± 0.48	4.80 ± 0.47	N.A.	82 ± 6.95	67 ± 4.78	N.A.
6	5.70 ± 0.72	6.90 ± 0.65	N.A.	60 ± 2.84	56 ± 1.98	N.A.
7	16.70 ± 1.50	18.30 ± 1.75	N.A.	7 ± 0.94	5 ± 0.46	N.A.
8	19.00 ± 1.85	20.00 ± 1.92	N.A.	3 ± 0.45	5 ± 0.46	N.A.
9	3.65 ± 0.29	4.20 ± 0.44	N.A.	86 ± 3.09	83 ± 4.65	N.A.
10	14.18 ± 1.36	16.10 ± 1.40	N.A.	9 ± 0.47	6 ± 0.45	N.A.

N.A. is no activity.
[a)] The percentage changes as compared with control untreated cancer cells (DMSO treated).

pyrazole structure (compounds **4**, **6**, **7**, **8**, and **10**) decreased the anticancer activity.

Experimental

Chemistry

All melting points are uncorrected and were measured using an Electrothermal IA 9100 apparatus, Shimadzu (Japan). The IR spectra (KBr) were recorded on a Perkin–Elmer 1650 spectrophotometer, National Research Centre, Cairo, Egypt. Microanalytical data were measured by Vario El-Mentar apparatus, Organic Microanalysis Section, National Research Centre, Cairo, Egypt, and the results were found to be in agreement with the calculated values (±0.3). ^{1}H and ^{13}C NMR spectra were determined on a Jeol 300 MHz in dimethyl sulfoxide (DMSO-d_6), National Research Centre, and the chemical shifts were expressed in ppm relative to TMS as internal reference. Mass spectra were recorded on 70 eV EI Ms-QP 1000 EX (Shimadzu, Japan), National Research Centre, Cairo, Egypt. Compound **1** (mp 167°C; literature mp 167–167.5°C) was prepared according to a reported method [38].

*N-Acetyl-N-(4-cyano-2-p-tolyl-2H-pyrazol-3-yl)acetamide **2***

A solution of compound **1** (0.002 mol) in acetic anhydride (10 mL) was stirred at room temperature for 3 h. The solvent was removed under reduced pressure and the obtained solid was recrystallized from dry ethanol to give compound **2** in 71% yield; mp 150–152°C; IR (KBr): ν 2233 (C≡N), 1741, 1720 (C=O) cm^{-1}; ^{1}H NMR (DMSO-d_6): δ 8.19 (d, 2H, Ar-H, J = 8.6 Hz), 7.60 (s, 1H, C_3-H), 7.33 (d, 2H, Ar-H, J = 8.6 Hz), 3.25 (s, 3H, CH_3), 3.24 (s, 3H, CH_3), 2.42 (s, 3H, CH_3); ^{13}C NMR (DMSO-d_6): δ 158.20 (2C=O), 149.25 (C-5); 140.81 (C-3); 134.17, 130.38, 127.16, 113.51 (Ar-C), 120.7 (C≡N), 95.72 (C-4); 25.55 (CH_3), 20.19 (CH_3), 20.15 (CH_3); MS, m/z (%): 282 (M^+, 80); Anal. calcd. for $C_{15}H_{14}N_4O_2$: C, 63.82; H, 5.0; N, 19.85. Found: C, 63.98; H, 4.92; N, 19.89.

*6-Methyl-1-p-tolyl-1,5-dihydro-pyrazolo[3,4-d]pyrimidin-4-one **3***

Method A: A mixture of compound **1** (0.01 mol) in acetic anhydride/glacial acetic acid (20 mL, 1:1) was heated under reflux for 10 h. The reaction mixture was cooled and poured into ice water; the formed solid was filtered off, dried, and recrystallized from dioxane to give compound **3** in 84% yield.

Method B: A mixture of compound **4** (0.01 mol) in hydrochloric acid/glacial acetic acid (20 mL, 1:3) was heated under reflux for 5 h. The reaction mixture was poured into ice water; the formed solid was filtered off, washed several times with water, dried, and recrystallized from dioxane to give compound **3** in 89% yield. mp 210–212°C; IR (KBr) ν 3220 (NH), 1675 (C=O) cm^{-1}; ^{1}H NMR (DMSO-d_6): δ 9.42 (s, 1H, NH, D_2O exchangeable), 8.20 (d, 2H, Ar-H, J = 8.6 Hz), 7.62 (s, 1H, C_3-H), 7.34 (d, 2H, Ar-H, J = 8.6 Hz), 2.64 (s, 3H, CH_3), 2.41 (s, 3H, CH_3); ^{13}C NMR (DMSO-d_6): δ 165.10 (C=O), 154.84 (C-6), 141.92 (C-3); 122.23 (C-7a); 102.32 (C-3a); 134.18, 130.39, 127.17, 113.52 (Ar-C), 24.19 (CH_3), 25.40 (CH_3); MS, m/z (%): 240 (M^+, 67); Anal. calcd. for $C_{11}H_{10}N_4$: C, 64.99; H, 5.03; N, 23.32. Found: C, 65.12; H, 4.90; N, 23.39.

*6-Methyl-1-p-tolyl-1,5-dihydro-pyrazolo[3,4-d][1,3]oxazin-4-one **4***

A mixture of compound **1** (0.01 mol) in hydrochloric acid/glacial acetic acid (20 mL, 1:3) was stirred at room temperature for 5 h. The reaction mixture was poured into icewater; the formed solid was filtered off and washed several times with water to give compound **4** in 78% yield; mp 180–182°C; IR (KBr) ν 3210 (NH) cm^{-1}; ^{1}H NMR (DMSO-d_6): δ 12.10 (s, 1H, NH, D_2O exchangeable), 8.22 (d, 2H, Ar-H, J = 8.6 Hz), 7.60 (s, 1H, C_3-H), 7.32 (d, 2H, Ar-H, J = 8.6 Hz), 2.42 (s, 3H, CH_3), 2.32 (s, 3H, CH_3). ^{13}C NMR (DMSO-d_6): δ 160.10 (C=NH), 155.99 (C-6), 140.98 (C-3); 122.22 (C-7a); 104.51 (C-3a); 134.19, 130.40, 127.16, 113.42 (Ar-C), 24.20 (CH_3), 25.41 (CH_3); MS, m/z (%): 240 (M^+, 59); Anal. calcd. for $C_{13}H_{12}N_4O$: C, 64.99; H, 5.03; N, 23.32. Found: C, 64.95; H, 4.95; N, 23.35.

*N-(4-Cyano-2-p-tolyl-2H-pyrazol-3-yl)formamide **5***

A solution of compound **1** (0.002 mol) in formic acid (10 mL, 85%) was stirred at room temperature for 6 h. The reaction mixture was poured into water; the formed solid was filtered off, washed several times with water, dried, and recrystallized from ethanol to give compound **5** in 71% yield; mp 150–152°C; IR (KBr): ν 2233 (C≡N), 1723 (C=O) cm^{-1}; ^{1}H NMR (DMSO-d_6): δ 8.45 (bs, 1H, NH, D_2O exchangeable), 8.26 (s, 1H, CHO), 8.20 (d, 2H, Ar-H, J = 8.6 Hz), 7.61 (s, 1H, C_3-H), 7.33 (d, 2H, Ar-H, J = 8.6 Hz), 2.42 (s, 3H, CH_3). ^{13}C NMR (DMSO-d_6): δ 161.30 (C=O), 148.05 (C-5); 139.72 (C-3); 134.09, 130.29, 127.09, 113.35 (Ar-C), 119.8 (C≡N), 94.79 (C-4); 25.55 (CH_3); MS, m/z (%): 226 (M^+, 62); Anal. calcd. for $C_{12}H_{10}N_4O$: C, 63.71; H, 4.46; N, 24.77. Found: C, 63.65; H, 4.49; N, 24.79.

*1-(p-Tolyl)-1,5-dihydro-1H-pyrazolo[3,4-d]pyrimidin-4-one **6***

Method A: A solution of compound **1** (0.01 mol) in formic acid (20 mL, 85%) was heated under reflux for 10 h. The reaction mixture was cooled and poured into water, and the formed solid was filtered off, dried, and recrystallized from dioxane to give compound **6** in 76% yield.

Method B: Compound **5** (0.01 mol) was heated under reflux in formic acid (20 mL, 85%) for 4 h. The reaction mixture was cooled and poured into water. The formed solid was filtered off, dried, and recrystallized from dioxane to give compound **6** in 89% yield. mp 275–277°C; IR (KBr) ν 3150 (NH), 1674 (C=O) cm^{-1}; ^{1}H NMR (DMSO-d_6): δ 10.10 (s, 1H, NH, D_2O exchangeable), 8.55 (s, 1H, C_6-H), 8.20 (d, 2H, Ar-H, J = 8.6 Hz), 7.59 (s, 1H, C_3-H), 7.34 (d, 2H, Ar-H, J = 8.6 Hz), 2.43 (s, 3H, CH_3); ^{13}C NMR (DMSO-d_6): δ 167.20 (C=O), 158.12 (C-6), 144.22 (C-3); 123.01 (C-7a); 103.22 (C-3a); 133.12, 130.59, 127.11, 113.21 (Ar-C), 24.18 (CH_3); MS, m/z (%): 226 (M^+, 90); Anal. calcd. for $C_{12}H_{10}N_4O$: C, 63.71; H, 4.46; N, 24.77. Found: C, 63.65; H, 4.50; N, 24.74.

*6-Mercapto-1-p-tolyl-1,5-dihydro-pyrazolo[3,4-d]-pyrimidine-4-thione **7***

A solution of compound **1** (0.01 mol) and 20% potassium hydroxide solution (potassium hydroxide 1.68 g, 7 mL water) in dimethylsulfoxide (20 mL) was stirred and carbon disulfide (0.03 mol) was added in several portions for 30 min. After 1 h at room temperature, the precipitate was collected, washed several times with water, dried, and recrystallized from dimethyl formamide to give compound **7** in 69% yield; mp 255–257°C; IR (KBr) ν 3100 (NH) cm^{-1}; ^{1}H NMR (DMSO-d_6): δ 11.30 (s, 1H, NH, D_2O exchangeable), 8.21 (d, 2H, Ar-H, J = 8.6 Hz), 7.60 (s, 1H, C_3-H), 7.34 (d, 2H, Ar-H, J = 8.6 Hz), 4.50 (s, 1H, SH, D_2O exchangeable), 2.41 (s, 3H, CH_3); ^{13}C NMR (DMSO-d_6): δ 170.23 (C=S), 159.52 (C-6), 143.12 (C-3); 123.25 (C-7a); 104.62 (C-3a); 134.19, 130.30, 127.09, 113.35 (Ar-C), 24.19 (CH_3); MS, m/z (%): 274 (M^+, 90); Anal. calcd. for

$C_{12}H_{10}N_4S_2$: C, 52.53; H, 3.67; N, 20.42; S, 23.37. Found: C, 52.42; H, 3.70; N, 20.48; S, 23.41.

7-(4-Methoxy-benzylidene)-4-thioxo-1-p-tolyl-1,4-dihydro-pyrazolo[3,4-d]thiazolo[3,2-a]pyrimidin-6-one ***8***

A mixture of compound **7** (0.002 mol), chloroacetic acid (10 mol), 4-methoxybenzaldehyde (0.002 mol), and anhydrous sodium acetate was heated under reflux in glacial acetic acid (15 mL) and acetic anhydride (10 mL) for 5 h. The reaction mixture was cooled and poured into cold water (100 mL), the deposited precipitate was filtered off and recrystallized from dioxane to produce **8** in 56% yield; mp 250–252°C; ^{1}H NMR (DMSO-d_6): δ 8.36 (s, 1H, C_3-H), 8.20 (d, 2H, Ar-H, $J = 8.5$ Hz), 8.12 (s, 1H, =CH), 7.51–7.80 (m, 4H, Ar-H), 7.34 (d, 2H, Ar-H, $J = 8.5$ Hz), 3.92 (s, 3H, OCH_3), 2.41 (s, 3H, CH_3); MS, *m/z* (%): 432 (M^+, 45); Anal. calcd. for $C_{22}H_{16}N_4O_2S_2$: C, 61.09; H, 3.73; N, 12.95; S, 7.40. Found: C, 61.40; H, 3.72; N, 12.88; S, 7.46.

1-(4-Chlorophenyl)-3-(4-cyano-2-p-tolyl-2H-pyrazol-3-yl)-thiourea ***9***

A mixture of compound **1** (0.002 mol), *p*-chlorophenyl isothiocyanate (0.002 mol), and triethylamine (2 drops) in dimethyl formamide (10 mL) was stirred at room temperature for 3 h. The reaction mixture was poured onto ice water; the deposited solid was filtered off and washed several times with water to give compound **9** in 66% yield; mp 152–154°C; IR (KBr) ν 3359, 3176 (NH), 2215 (C≡N) cm^{-1}; ^{1}H NMR (DMSO-d_6): δ 8.28–8.21 (m, 4H, Ar-H), 7.90 (s, 1H, NH, D_2O exchangeable), 7.62 (s, 1H, C_3-H), 7.41–7.33 (m, 4H, Ar-H), 6.50 (s, 1H, NH, D_2O exchangeable), 2.41 (s, 3H, CH_3); MS, *m/z* (%): 369 (M^++2, ^{37}Cl, 39.98), 367 (M^+, ^{35}Cl, 100); Anal. calcd. for $C_{18}H_{14}ClN_5S$: C, 58.77; H, 3.84; N, 19.04; S, 8.72. Found: C, 58.52; H, 3.79; N, 19.18; S, 8.49.

5-(4-Chlorophenyl)-4-imino-1-(4-methylphenyl)-1,4,5,7-tetrahydro-6H-pyrazolo[3,4-d]pyrimidin-6-thione ***10***

Method A: A mixture of **9** (0.002 mol) and triethylamine (2 drops) in dimethyl formamide (10 mL) was refluxed for 5 h. The reaction mixture was poured onto ice water; the deposited solid was filtered off and washed several times with water to give compound **10** (68% yield).

Method B: A mixture of compound **1** (0.002 mol) and *p*-chlorophenyl isothiocyanate (0.002 mol) in dimethyl formamide (20 mL) was refluxed for 6 h. The reaction mixture was poured onto ice water and the solid formed was separated by filtration and washed several times with water to give compound **10** (61% yield). mp 175–177°C; IR (KBr) ν 3272, 3190 (2NH) cm^{-1}; ^{1}H NMR (DMSO-d_6): δ 10.45 (s, 1H, NH, D_2O exchangeable), 8.41 (s, 1H, C_3-H), 8.20–8.29 (m, 4H, Ar-H), 7.4–7.3 (m, 4H, Ar-H), 6.42 (s, 1H, NH, D_2O exchangeable), 2.42 (s, 3H, CH_3); ^{13}C NMR (DMSO-d_6): δ 171.95 (C=S), 160.99 (C-4), 143.01 (C-3); 123.25 (C-7a); 104.62 (C-3a); 134.20, 132.12, 129.05, 128.60, 128.12, 127.29, 126.83, 125.21 (Ar-C), 24.25 (CH_3); MS, *m/z* (%):369 (M^++2, ^{37}Cl, 39.98), 367 (M^+, ^{35}Cl, 100); Anal. calcd. for $C_{18}H_{14}ClN_5S$: C, 58.77; H, 3.84; N, 19.04; S, 8.72. Found: C, 58.65; H, 3.81; N, 18.98; S, 8.62.

Anticancer evaluation

Chemicals

Fetal bovine serum (FBS) and L-glutamine were obtained from Gibco Invitrogen Company (Scotland, UK). Dulbecco's modified Eagle's (DMEM) medium was provided from Cambrex (NJ, USA). DMSO, doxorubicin, penicillin, and streptomycin were obtained from Sigma Chemical Company (Saint Louis, MO, USA). The level of uPA protein was determined using Assay Max human urokinase (uPA) ELISA kit (Assaypro, USA).

Cell lines and culturing

Anticancer activity screening was performed for the tested compounds utilizing three different human tumor cell lines including breast cancer cell line MCF-7, liver cancer cell line HepG2, and lung carcinoma cell line A549 obtained from the American Type Culture Collection (Rockville, MD, USA). The tumor cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (GIBCO), penicillin (100 U/mL), and streptomycin (100 μg/mL) at 37°C in humidified atmosphere containing 5% CO_2. Cells at a concentration of 0.50×10^6 were grown in a 25 cm^2 flask in 5 mL of complete culture medium.

In vitro cytotoxicity assay

The cytotoxicity activity was measured *in vitro* using the Sulfo-Rhodamine-B stain (SRB) assay according to the previously reported standard procedure [40]. Cells were inoculated in 96-well microtiter plate (10^4 cells/well) for 24 h before treatment with the tested compounds to allow attachment of cells to the wall of the plate. Test compounds were dissolved in DMSO at 1 mg/mL immediately before use and diluted to the appropriate volume just before addition to the cell culture. Different concentrations of tested compounds and doxorubicin were added to the cells. Triplicate wells were prepared for each individual dose. Monolayer cells were incubated with the compounds for 48 h at 37°C and in atmosphere of 5% CO_2. After 48 h, cells were fixed, washed, and stained for 30 min with 0.4% w/v SRB dissolved in 1% acetic acid. Unbound dye was removed by four washes with 1% acetic acid, and attached stain was recovered with Tris–EDTA buffer. Color intensity was measured in an ELISA reader. The relation between surviving fraction and drug concentration was plotted to get the survival curve for each cell line after the specified time. The concentration required for 50% inhibition of cell viability (IC_{50}) was calculated, and the results are given in Table 1. The results were compared to the antiproliferative effects of the reference control doxorubicin.

Determination of the level of uPA protein expression

The level of uPA protein expression was determined using Assay Max human urokinase (uPA) ELISA kit (Assaypro, USA) according to manufacturer's instructions. The prepared compounds as well as standard drug doxorubicin were incubated for 48 h with MCF7, HepG2, and A549 cells at a concentration of 1/10 of the IC_{50} values of each compound shown in Table 1.

After 48 h from compounds treatment, medium was collected and centrifuged at $2000 \times g$ for 10 min to remove cellular debris; 50 μL of the cell extract was added per well and incubated for 2 h. Wells were washed with 200 μL of wash buffer; then 50 μL of biotinylated uPA antibody was added to each well and incubated for 1 h at 25°C. After washing, plates were incubated with 50 μL of streptavidin–peroxidase conjugate per well and incubated for 30 min; then the microplate was washed as described above; 50 μL of chromogen substrate was added per well and incubated for about 10 min or till the optimal blue color density developed; 50 μL of stop solution was

added to each well. The color changed from blue to yellow. The absorbance was read on a microplate reader at a wavelength of 450 nm immediately, the concentrations of uPA in the samples were determined, and the percentage of uPA inhibition for each compound was calculated as compared with control cancer cells (DMSO treated).

Statistical analysis

The results were reported as mean ± SE. Statistical differences were analyzed according to one-way ANOVA test Student's *t*-test wherein the differences were considered to be significant at $p < 0.05$.

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