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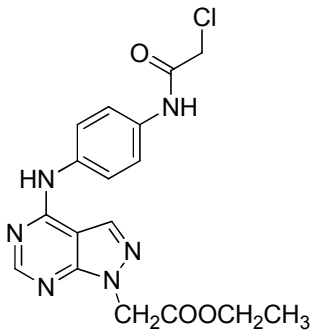
Graphical Abstract



5e

Compound **5e** possessed the best antitumor activity than 17AAG and allopurinol.

Synthesis and biological evaluation of pyrazolo[4,3-d] pyrimidine analogues

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ABSTRACT

A series of pyrazolo[3,4-d]pyrimidine analogues **3**, **4**, **5a**–**5f**, **6a**–**6f** with various amines and ester groups at C-4 and N-1 were synthesized and evaluated for antitumour activity. They were also evaluated for xanthine oxidase inhibitory activity, with most compounds having no significant impact. Compound **5e** had the strongest activity against human hepatoma carcinoma cells 7402 and 7221, with half-maximal inhibitory concentration values of 4.55 and 6.28, respectively. Structure–activity relationship studies indicate that chlorine atoms in the structure of 4-((4-(substituted amides)phenyl)amino pyrazolo[4,3-d]pyrimidine analogues is crucial for antitumour activity.

Keywords: pyrazolo[3,4-d]pyrimidines; XOD; antitumour; 17AAG; allopurinol.

1. Introduction

The term cancer encompasses a wide range of types such as lung cancer, colon cancer, and the more obscure acute leukaemia. Malignant cancers are very common and are the second largest cause of death in the West after cardiovascular disease. It is one of the major challenges of this century and is a concern for medical communities all over the world. The diversity of tumour types and their great similarity to normal cells are the main obstacles that prevent the discovery of a cure [1-6].

In the last decade or so, researchers have reported that purine derivatives of allopurinol (Fig. 1) have a certain inhibitory activity towards tumour cells [7]; they can inhibit heat shock protein 90 (Hsp90), which is involved in the degradation of somatic proteins. In addition, allopurinol is a structural isomer of hypoxanthine (a naturally occurring purine in the body) and can be used as inhibitor of the enzyme xanthine oxidase (XOD) [8-12]. Hsp90 is an important target for antitumour drugs owing to its effect on proteins, which can promote the growth and metastasis of tumours[13,14]. Purine derivative PU3 (Fig. 1), similar to geldanamycin (GA, Fig. 1), is the first purine compound discovered that can inhibit Hsp90 biological activity. Researchers have also found that PU24F-Cl has a stronger affinity to Hsp90 than PU3, and it has higher solubility than 17-AAG [15,16](Fig. 1). Although they are very structurally similar; the hydroxyl being replaced by an amino in the pyrimidine ring, and N-1 and C-2 being replaced by alkyl and 3,4,5-methoxy-1-benzyl in the pyrazole ring, respectively.

In this study, we carried out chemical modification of purine by introduction of a

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pyrazole ring instead of an imidazole ring to provide a specific binding mode different from that of allopurinol analogues [17-22]. We synthesized pyrazolo[3,4-d]pyrimidines analogues **3, 4, 5a**–**5f**, **6a**–**6f** by introducing various substituent groups at the C-4 and N-1 positions of the pyrazolopyrimidine ring (Scheme 1). The aim is to produce a potent and selective Hsp90 inhibitor. XOD inhibitory activity was evaluated, and all these new compounds were also tested for antitumour activity against the human hepatoma carcinoma cells 7402 and 7221 using the half-maximal inhibitory concentration (IC_{50}) values.

2. Results and Discussion

2.1. Synthesis

The general synthetic route for preparation of two series of Hsp90 inhibitors (**5a**–**5f** and **6a**–**6f**) is shown in Scheme 1. In the pyrazolopyrimidine ring system, the chloro substituent, as a leaving group, was introduced at C-4, which was the most reactive site for nucleophilic attack. Heating commercially available allopurinol (**1**) with excess *N*,*N*-dimethylaniline and phosphorus oxychloride gave an intermediate product **2**, which was treated with ethyl bromoacetate to afford the corresponding ethyl 2-(4-chloro-1H-pyrazolo[3,4-d]pyrimidin-1-yl)acetate (**3**). Reacting **3** with p-phenylenediamine in acetonitrile at room temperature yielded compound **4**. Amination of **4** with sulfonyl chloride or chloride gave target compounds **5a–5f**, Scheme 1.

2.2. Effect on XOD inhibitory activity

Inhibition of the XOD-catalyzed conversion of xanthine to uric acid by all 1-*N*-alkyl-4-*N*-substituted amino allopurinol derivative inhibitors was evaluated and compared with the standard inhibitor allopurinol. Enzyme activity was spectrophotometrically monitored by measuring uric acid formation at 290 nm. This was done with a saturated concentration of xanthine (20 μM) as the substrate in 1 mL of 200 mM phosphate buffer at pH 7.5 and at 25 °C. All samples were tested for XOD inhibitory activity at different concentrations (5, 10, 15 and 20 μM). The test results are listed in Table 1.

On observing the data in Table 1, there seems to be no correlation between XOD inhibitory activity and the substituent size at C-4 and N-1 of the pyrazolopyrimidine skeleton, as there is no significant difference from the standard allopurinol compound. Most compounds did not show any significant XOD inhibitory activity. Compounds **5d**, **5f**, **6e** and **6f** exhibited potent XOD inhibitory activity against the target organisms, possibly because of the structural differences i.e. 4-hydroxyl versus amino. A previous literature report has shown that allopurinol derivatives such as 4-amino-substituted allopurinol have a lower XOD inhibitory activity than 4-hydroxyl-substituted allopurinol [9]. The results of these structure–activity studies suggest that the structure of purine is crucial for XOD inhibitory activity.

2.3. Effect on antitumour activity

The synthesized compounds (**3–6**; Scheme 1) were subjected to the human cell lines screening assay for evaluation of their in vitro antitumour activity. A single high dose (64 μM) of the test compounds were used in the full NCI two cell lines panel

assay which includes human hepatoma carcinoma cells 7402 and 7221. The data were reported as a mean graph of the percent growth of treated cells and presented as IC_{50} values, Table 2. The obtained results of the tested allopurinol derivatives **3**, **4**, **5a**, **5b**, **5c**, **5d**, **5f**, **6a**, **6b**, **6c**, **6d**, **6e**, **6f** showed IC_{50} values >60 μg/ml, a distinctive potential pattern of selectivity, as well as broad-spectrum antitumour activity. Compound **5e** showed potency against human hepatoma carcinoma cells 7402 and 7221 lines with IC_{50} values of 7.27 and 3.51, respectively. It also has a higher antitumour activity compared with 17AAG. This difference indicates that **5e** may exert its cell growth inhibitory effect through partial disruption or suppressed dynamics of microtubules or even through interactions with other cellular targets.

2.4. Structure–activity correlation

Structure–activity correlation, based on the number of cell lines that showed sensitivity toward each of the synthesized individual compounds, revealed that ethyl 2-(4-((4-(substituted amides)phenyl)amino)-1H-pyrazolo[3,4-d]pyrimidin-1-yl) acetate (**5**) is a more active antitumour agent than 2-(4-((4-(4-substituted amides) phenyl)amino)-1H-pyrazolo[3,4-d] pyrimidin-1-yl)acetic acid (**6**). It is possible that the introduction of an ester group may increase the lipid/water partition coefficient. As previously reported for PU_3 containing an amino group at position 4 and an ethyl group at position 1 (Fig. 1), amino substitution at position 4 and ester group substitution at position 1 were also observed in compound **4**. The presence of an ester group at position N1, rather than a carboxylic acid group, proved essential for activity. Ethyl 2-(4-((4-aminophenyl) amino)-1H-pyrazolo[3,4-d]pyrimidin-1-yl)acetate (**4**) is a more active antitumour agent than ethyl 2-(4-chloro-1H-pyrazolo[3,4-d]pyrimidin-1-yl)acetate (**3**). This suggested that the presence of a nitrogen atom (aminoanilino group) at position C4 enhances the antitumour effect. Among the derivatives of **5** (**5a**–**5c**), the substituted benzenesulfonamide pyrazolo[4,3-d]pyrimidine derivative showed more potent activity than the unsubstituted derivative. Moreover, among the derivatives of **5** (**5d**–**5f**), **5e** and **5f** are small molecules of fatty acid amides. The negative inductive effect of **5f** reduces the density of the electron cloud. Although the presence of a strong negative halogen atom in **5e** exerts a strong electron-withdrawing effect, the presence of chlorine atoms in **5e** can enhance the activity toward the cell lines.

3. Conclusion

Among the compounds that displayed stronger antitumour activity, only **5e** exhibited a reasonable antitumour activity. Pyrazolo[4,3-d]pyrimidines were found to have a much weaker XOD inhibitory activity than the standard allopurinol. On the other hand, ethyl 2-(4-((4-(2-chloroacetamido)phenyl)amino)-1H-pyrazolo [3,4-d]pyrimidin-1-yl) acetate (**5e**) (Fig. 2) exhibited an antitumour activity higher than 17AAG and allopurinol. Therefore, the unique dual specificity of **5e**, which displays an antitumour activity more potent than conventional drugs, represents an attractive starting point for further optimization. Further studies on the antitumour mechanisms of **5e** are underway.

4. Experiment

Melting Point were measured with a micromelting point tester and are uncorrected.

^{1}H and ^{13}C NMR spectra were obtained in DMSO-d_6 or $CDCl_3$ solutions on a Bruker AVII-400 spectrometer operating at 400 and 100 MHz, respectively. High-resolution mass spectra were obtained on a Waters Q-TOF-Premiter instrument (TOF-MS). Infrared (IR) spectra were obtained by Perkin-Eliner 16PC-FT infrared spectrometer. Thin layer chromatography (TLC) was employed to routinely monitor the reaction samples and confirm the homogeneity of the analytical samples by using Kieselgel 60F254 (0.25 mm) silica gel TLC aluminum sheets. Chromatography was carried out using Merck silica yel 60, 200-300 mesh. All reagents were commercially available and were used without further purification unless otherwise indicated. Allopurinol and xanthine oxidase were purchased from Sigma. Dimethylsulfoximine (DMSO) was purchased from Amresco.

4.1. Chemistry

*4.1.1. 4-chloro-1H-pyrazolo[3,4-d]pyrimidine (**2**)*

A mixture of Allopurinol **1** (2.00 g, 14.69 mmol) and N,N-dimethylaniline (2.00 g, 16.52 mmol) was stirred in $POCl_3$ (25 mL) at 80 °C for 2 h. The reaction mixture was diluted with water (35 mL), and extracted with ethyl acetate. The organic layer was washed with water and the organic phase was concentrated to dryness, and the residue was purified by column chromatography on silica gel using 4:1.5 petroleum ether/ ethyl acetate as the eluent to give **2**.

*4.1.2. ethyl 2-(4-chloro-1H-pyrazolo[3,4-d]pyrimidin-1-yl)acetate (**3**)*

To a solution of 4-chloro-1H-pyrazolo[3,4-d]pyrimidine **2** (0.81 g, 5.26 mmol) in anhydrous DMF (15 mL) was added Triethylamine (1.78 g, 17.62 mmol). The mixture was stirred at room temperature for 30min. Then Ethylbromoacetate (1.06g, 6.39mmol) was added and the mixture was stirred at room temperature for 1 h and KI was added. The reaction mixture was subsequently diluted with water (45 mL), acidified with HCl and extracted with ethyl acetate. Finally, the organic layer was washed with water and the organic phase was concentrated to dryness, and the residue was purified by column chromatography on silica gel using 10:1 petroleum ether/ ethyl acetate as the eluent to give **3**: white powder; mp 83-84□; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 8.91 (s, 1H, CH), 8.58 (s, 1H, CH), 5.45 (s, 2H, CH_2), 4.17 (dd, J = 7.10 Hz, J = 14.22 Hz, 2H, CH_2), 1.20 (t, J = 7.10 Hz, 3H, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6) 167.90, 156.53, 151.47, 151.09, 135.32, 106.29, 61.80, 48.86, 14.36; IR (KBr, v, cm^{-1}): 3458, 3116, 2992, 2938, 1734, 1591, 1556, 1486, 1404, 1357, 1250, 1218, 1188, 1134, 1019, 950, 861, 786, 713, 568; HRMS (ESI) calcd for $C_9H_9ClN_4O_2$ $[M + H]^+$, 240.0414; found, 241.0492.

*4.1.3. ethyl 2-(4-((4-aminophenyl)amino)-1H-pyrazolo[3,4-d]pyrimidin-1- yl)acetate (**4**)*

To a solution of ethyl 2-(4-chloro-1H-pyrazolo[3,4-d]pyrimidin-1-yl)acetate **3** (1.73 g, 7.21 mmol) in acetonitrile (5 mL) was added benzene-1,4-diamine (0.78 g, 7.21 mmol). The mixture was stirred at 80 □ for 3 h. The reaction mixture was concentrated to dryness, and the residue was purified by recrystallization using ethyl acetate to give **4**: mp 179.8–181.9□; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 9.76 (br, 1H,

NH), 8.27 (s, 2H, CH), 7.37 (s, 2H, ArH), 6.62 (s, 2H, ArH), 5.02–5.18(m, 4H), 4.14 (dd, J = 7.04 Hz, J = 14.16 Hz, 2H, CH_2), 1.19 (t, J = 7.08 Hz, 3H, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6) 167.80, 155.73, 154.30, 153.61, 132.52, 127.41, 123.34, 113.94, 100.76, 61.18, 47.80, 13.94; IR (KBr, v, cm^{-1}): 3476, 3381, 3200, 2940, 1735, 1591, 1514, 1440, 1329, 1291, 1254, 1212, 1019, 915, 795, 740, 706, 603, 516; HRMS (ESI) calcd for $C_{15}H_{16}N_6O_2$ [M + H]$^+$, 312.1335; found, 313.1439.

*4.1.6. ethyl 2-(4-((4-(4-bromophenylsulfonamido)phenyl)amino)-1H-pyrazolo[3,4-d] pyrimidin-1-yl)acetate (**5a**)*

To a solution of ethyl 2-(4-((4-aminophenyl)amino)-1H-pyrazolo[3,4-d]pyrimidin-1-yl)acetate **4** (0.36 g, 1.15 mmol) in THF(10 mL) was added NaH (0.069 g, 2.88 mmol) at 0 °C and stirred for 30 min. And then 4-bromobenzene-1-sulfonyl chloride (0.34 g, 1.34 mmol) was added and stirred at 0 °C for 2 h. The reaction mixture was diluted with water (15 mL), and extracted with dichloromethane. The organic layer was washed with water and the organic phase was concentrated to dryness, and the residue was purified by column chromatography on silica gel using 2:1 petroleum ether/ acetone as the eluent to give **5a**: 54%; mp 201.5–203 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 10.28 (br, 1H, NH), 10.09 (br, 1H, NH), 8.38 (s, 1H, CH), 8.22 (s, 1H, CH), 7.79 (d, J = 8.60 Hz, 2H, ArH), 7.65–7.71 (m, 4H, ArH), 7.10 (d, J = 8.88 Hz, 2H, ArH), 5.23(s, 2H, CH_2), 4.15 (dd, J = 7.12 Hz, J = 14.24 Hz, 2H, CH_2), 1.19(t, J = 7.10 Hz, 3H, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6) 168.20, 155.99, 154.67, 154.05, 139.16, 136.31, 133.24, 132.79, 129.19, 127.22, 122.63, 122.16, 101.32, 61.71, 48.41, 14.44; IR (KBr, v, cm^{-1}): 3369, 3221, 2927, 2854, 1747, 1622, 1576, 1508, 1435, 1321, 1291, 1209, 1157, 1091, 1026, 917, 793, 742, 655, 606, 549, 419; HRMS (ESI) calcd for $C_{21}H_{19}BrN_6O_4S$ [M + H]$^+$, 530.0372; found, 531.0442.

*4.1.7. ethyl 2-(4-((4-(4-nitrophenylsulfonamido)phenyl)amino)-1H-pyrazolo[3,4-d]pyrimidin-1-yl)acetate (**5b**)*

To a solution of ethyl 2-(4-((4-aminophenyl)amino)-1H-pyrazolo[3,4-d]pyrimidin-1-yl)acetate **4** (0.36 g, 1.15 mmol) in THF(10mL) was added NaH (0.069 g, 2.88 mmol) at 0 °C and stirred for 30 min. And then 4-nitrobenzene-1-sulfonyl chloride (0.30 g, 1.34 mmol) was added and stirred at 0 °C for 2 h. The reaction mixture was diluted with water (15 mL), and extracted with dichloromethane. The organic layer was washed with water and the organic phase was concentrated to dryness, and the residue was purified by column chromatography on silica gel using 2:1 petroleum ether/ acetone as the eluent to give **5b**: 48%; mp182–183 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 10.53 (br, 1H,

NH), 10.10 (br, 1H, NH), 8.37–8.41(m, 3H), 8.20 (s, 1H, CH), 8.00 (d, *J* = 8.88 Hz, 2H, ArH), 7.72 (d, *J* = 8.72 Hz, 2H, ArH), 7.12(d, *J* = 8.88 Hz, 2H, ArH), 5.22 (s, 2H, CH_2), 4.15 (dd, *J* = 7.12 Hz, *J* = 14.24 Hz, 2H, CH_2), 1.19 (t, *J* = 7.12 Hz, 3H, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6) 168.18, 155.94, 154.66, 154.03, 150.25, 145.33, 136.66, 132.87, 132.68, 128.80, 125.04, 122.49, 101.31, 61.72, 48.38, 14.41; IR (KBr, *v*, cm^{-1}): 3390, 3253, 3108, 1734, 1620, 1573, 1529, 1481, 1379, 1346, 1213, 1166, 1090, 1015, 919, 857, 792, 737, 607, 547, 464; HRMS (ESI) calcd for $C_{21}H_{19}N_7O_6S$ $[M + H]^+$, 497.1118; found, 498.1199.

*4.1.8. ethyl 2-(4-((4-(phenylsulfonamido)phenyl)amino)-1H-pyrazolo[3,4-d]pyrimidin -1-yl)acetate (**5c**)*

To a solution of ethyl 2-(4-((4-aminophenyl)amino)-1H-pyrazolo[3,4-d]pyrimidin-1-yl)acetate **4** (0.36 g, 1.15 mmol) in THF(10 mL) was added NaH(0.069 g, 2.88 mmol) at 0 ℃ and stirred for 30min.And then benzenesulfonyl chloride (0.24 g, 1.34 mmol) was added and stirred at 0 ℃ for 2 h. The reaction mixture was diluted with water (15 mL), and extracted with dichloromethane. The organic layer was washed with water and the organic phase was concentrated to dryness, and the residue was purified by column chromatography on silica gel using 2:1 petroleum ether/ acetone as the eluent to give **5c**: 57%; mp224–226 °C;^{1}H NMR (400 MHz, DMSO-d_6) *δ*: 10.21 (br, 1H, NH), 10.06 (br, 1H, NH), 8.37 (s, 1H, CH), 8.20 (s, 1H, CH), 7.76 (d, *J* = 7.12 Hz, 2H, ArH), 7.54–7.67(m, 5H, ArH), 7.11 (d, *J* = 8.84 Hz, 2H, ArH), 5.22 (s, 2H, CH_2), 4.14 (dd, *J* = 7.12 Hz, *J* = 14.20 Hz, 2H, CH_2), 1.19 (t, *J* = 7.10 Hz, 3H, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6) 167.72, 155.51, 154.23, 153.56, 139.43, 135.46, 133.20, 132.81, 132.38, 129.18, 126.67, 122.32, 121.30, 100.76, 61.21, 47.91, 13.95; IR (KBr, *v*, cm^{-1}): 3345, 3236, 2927, 2854, 1740, 1621, 1573, 1508, 1483, 1441, 1375, 1294, 1212, 1235, 1162, 1093, 1016, 909, 870, 783, 725, 657, 583, 547; HRMS (ESI) calcd for $C_{21}H_{20}N_6O_4S$ $[M + H]^+$, 452.1267; found, 453.1355.

*4.1.9. ethyl 2-(4-((4-benzamidophenyl)amino)-1H-pyrazolo[3,4-d]pyrimidin-1 -yl)acetate (**5d**)*

To a solution of ethyl 2-(4-((4-aminophenyl)amino)-1H-pyrazolo[3,4-d]pyrimidin-1-yl)acetate **4** (0.50 g, 1.6 mmol) in THF(13 mL) was added Triethylamine (0.50 g, 4.95 mmol) at 0 ℃ and stirred for 30min. And then benzoyl chloride (0.25 g, 1.79 mmol) was added and stirred at 0 ℃ for 2 h. The reaction mixture was diluted with water (15 mL), and extracted with dichloromethane. The organic layer was washed with water and the organic phase was concentrated to dryness, and the residue was purified by column chromatography on silica gel using 2:1 petroleum ether/ acetone as the eluent to give **5d**: 59%; mp 233–235□; ^{1}H NMR (400 MHz, DMSO-d_6) *δ*: 10.30 (br, 1H, NH),

10.15 (br, 1H, NH), 8.42 (s, 1H, CH), 8.27 (s, 1H, CH), 7.97 (d, *J* = 7.72 Hz, 2H, ArH), 7.78–7.83 (m, 4H, ArH), 7.52–7.62 (m, 3H, ArH), 5.24 (s, 2H, CH_2), 4.16 (dd, *J* = 7.10 Hz, *J* = 14.22 Hz, 2H, CH_2), 1.20 (t, *J* = 7.08 Hz, 3H, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6) 168.25, 165.83, 156.11, 154.88, 154.11, 135.45, 135.12, 132.98, 131.94, 128.82, 128.09, 122.30, 121.25, 101.29, 61.72, 48.41, 14.45; IR (KBr, *v*, cm^{-1}): 3328, 3268, 3165, 2995, 2933, 1948, 1893, 1743, 1631, 1587, 1544, 1514, 1488, 1432, 1381, 1317, 1303, 1242, 1214, 1108, 1025, 919, 831, 784, 681, 524; HRMS (ESI) calcd for $C_{22}H_{20}N_6O_3$ $[M + H]^+$, 416.1597; found, 417.1672.

*4.1.10. ethyl 2-(4-((4-(2-chloroacetamido)phenyl)amino)-1H-pyrazolo[3,4-d] pyrimidin-1-yl) acetate (**5e**)*

To a solution of ethyl 2-(4-((4-aminophenyl)amino)-1H-pyrazolo[3,4-d]pyrimidin-1-yl)acetate **4** (0.50 g, 1.6 mmol) in THF(13 mL) was added Triethylamine (0.50 g, 4.95 mmol) at 0 ℃ and stirred for 30min. And then 2-chloroacetyl chloride (0.20 g, 1.79 mmol) was added and stirred at 0 ℃ for 2 h. The reaction mixture was diluted with water (15 mL), and extracted with dichloromethane. The organic layer was washed with water and the organic phase was concentrated to dryness, and the residue was purified by column chromatography on silica gel using 2:1 petroleum ether/ acetone as the eluent to give **5d**: 52%; mp 189.7–191.5□; ^{1}H NMR (400 MHz, DMSO-d_6) *δ*: 10.34 (br, 1H, NH), 10.14 (br, 1H, NH), 8.40 (s, 1H, CH), 8.26 (s, 1H, CH), 7.78 (d, *J* = 8.52 Hz, 2H, ArH), 7.62 (d, *J* = 8.96 Hz, 2H, ArH), 5.23 (s, 2H, CH_2), 4.26 (s, 2H, CH_2), 4.15 (dd, *J* = 7.10 Hz, *J* = 14.22 Hz, 2H, CH_2), 1.20 (t, *J* = 7.10 Hz, 3H, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6) 168.23, 164.89, 156.00, 154.78, 154.05, 135.26, 134.86, 132.97, 122.40, 120.27, 101.29, 61.72, 48.40, 44.04, 14.43; IR (KBr, *v*, cm^{-1}): 3627, 3265, 3197, 3110, 2992, 1742, 1667, 1572, 1513, 1434, 1294, 1209, 1095, 1019, 966, 919, 844, 785, 705, 656, 520; HRMS (ESI) calcd for $C_{17}H_{17}ClN_6O_3$ $[M + H]^+$, 388.1051; found, 389.1121.

*4.1.11. ethyl 2-(4-((4-acetamidophenyl)amino)-1H-pyrazolo[3,4-d]pyrimidin-1-yl)acetate (**5f**)*

To a solution of ethyl 2-(4-((4-aminophenyl)amino)-1H-pyrazolo[3,4-d]pyrimidin-1-yl)acetate **4** (0.50 g, 1.6 mmol) in THF(13 mL) was added Triethylamine (0.50 g, 4.95 mmol) at 0 ℃ and stirred for 30min. And then acetyl chloride (0.14 g, 1.79 mmol) was added and stirred at 0 ℃ for 2 h. The reaction mixture was diluted with water (15 mL), and extracted with dichloromethane. The organic layer was washed with water and the organic phase was concentrated to dryness, and the residue was purified by column chromatography on silica gel using 2:1 petroleum ether/ acetone as the eluent to give **5f**: 57%; mp 201–203□ ^{1}H NMR (400 MHz, DMSO-d_6) *δ*: 11.42 (br, 1H, NH), 10.25 (br, 1H, NH), 8.42–8.68 (m, 2H, CH), 7.61–7.72 (m, 4H, ArH), 5.31 (s, 2H,

CH_2), 4.18 (dd, *J* = 7.00 Hz, *J* = 14.08 Hz, 2H, CH_2), 2.08 (s, 3H, CH_3), 1.21 (t, *J* = 7.04 Hz, 3H, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6) 168.31, 167.45, 152.20, 134.17, 119.58, 100.19, 61.38, 48.32, 23.94, 13.97; IR (KBr, ν, cm^{-1}): 3359, 2921, 2851, 1746, 1665, 1524, 1396, 1295, 1211, 1096, 1022, 850, 791, 692, 596, 553; HRMS (ESI) calcd for $C_{17}H_{18}N_6O_3$ [M + H]$^+$, 354.1440; found, 355.1451.

*4.1.12. 2-(4-((4-(4-bromophenylsulfonamido)phenyl)amino)-1H-pyrazolo[3,4-d] pyrimidin-1-yl)acetic acid (**6a**)*

To a solution of ethyl 2-(4-((4-(4-bromophenylsulfonamido)phenyl)amino)-1H-pyrazolo[3,4-d] pyrimidin-1-yl)acetate **5a** (0.30 g, 0.57 mmol) in anhydrous anhydrous ethanol (5 mL) was added 1 N NaOH aqueous solution (1 mL). The mixture was stirred at room temperature 3 h. Then the reaction mixture was diluted with water (45 mL), acidified with HCl to 3 and extracted with ethyl acetate. The organic layer was washed with water and the organic phase was concentrated to dryness, and the residue was purified by recrystallization using anhydrous ethanol to give **6a**. In accordance with this method to get **6b–6f**. 49%; Pale yellow solid; mp 212–213□; ^{1}H NMR (400 MHz, DMSO-d_6) *δ*: 10.02 (br, 1H, NH), 8.28 (s, 1H, CH), 8.07 (s, 1H, CH), 7.65–7.72(m, *J* = 8.70 Hz, *J* = 21.46 Hz, 4H, ArH), 7.59 (s, 2H, ArH), 7.00 (d, *J* = 8.88 Hz, 2H, ArH), 4.72 (s, 2H, CH_2); ^{13}C NMR (100 MHz, DMSO-d_6) 175.45, 154.76, 154.19, 152.79, 139.19, 135.63, 133.27, 132.15, 131.03, 128.71, 126.39, 122.02, 121.65, 100.73, 50.62; IR (KBr, *v*, cm^{-1}): 3226, 1574, 1511, 1430, 1330, 1287, 1161, 1091, 1067, 1034, 924, 821, 788, 741, 706, 664, 602, 549; HRMS (ESI) calcd for $C_{19}H_{15}BrN_6O_4S$ [M + K]$^+$, 502.0059; found, 540.9611.

*4.1.13. 2-(4-((4-(4-nitrophenylsulfonamido)phenyl)amino)-1H-pyrazolo[3,4-d] pyrimidin-1-yl) acetic acid (**6b**)*

60%; Yellow solid; mp > 240 □; ^{1}H NMR (400 MHz, DMSO-d_6) *δ*: 9.82 (br, 1H, NH), 8.48 (s, 1H, CH), 8.28 (d, *J* = 8.80 Hz, 2H, ArH), 8.24 (s, 1H, CH), 7.95 (d, *J* = 8.84 Hz, 2H, ArH), 7.44 (s, 2H, ArH), 6.95 (d, *J* = 8.84 Hz, 2H, ArH), 4.71 (s, 2H, CH_2); ^{13}C NMR (100 MHz, DMSO-d_6) 174.91, 155.24, 154.68, 153.41, 149.81, 146.98, 135.92, 131.69, 128.67, 124.80, 122.31, 101.24, 50.95; IR (KBr, *v*, cm^{-1}): 3108, 1614, 1533, 1434, 1349, 1165, 1092, 1034, 926, 855, 789, 740, 664, 613, 551; HRMS (ESI) calcd for $C_{19}H_{15}N_7O_6S$ [M + K]$^+$, 469.0805; found, 508.0348.

*4.1.14. 2-(4-((4-(phenylsulfonamido)phenyl)amino)-1H-pyrazolo[3,4-d]pyrimidin -1-yl)acetic acid (**6c**)*

57%; Gray solid; mp 151–152□; ^{1}H NMR (400 MHz, DMSO-d_6) *δ*: 10.85 (br, 1H,

COOH), 10.25 (br, 2H, NH), 8.37 (s, 1H, CH), 8.23 (s, 1H, CH), 7.77 (d, *J* = 7.04 Hz, 2H, ArH), 7.54–7.68 (m, 5H, ArH), 7.12 (d, *J* = 8.88 Hz, 2H, ArH), 5.12 (s, 2H, CH_2); ^{13}C NMR (100 MHz, DMSO-d_6) 169.55, 154.93, 154.21, 153.55, 139.92, 135.45, 133.33, 133.23, 129.68, 127.18, 123.20, 121.75, 101.18, 48.61; IR (KBr, *v*, cm^{-1}): 3227, 2924, 2854, 1731, 1621, 1576, 1508, 1385, 1332, 1290, 1218, 1160, 1091, 1036, 922, 839, 810, 687, 582; HRMS (ESI) calcd for $C_{19}H_{16}N_6O_4S$ $[M + H]^+$, 424.0954; found, 425.1029.

*4.1.15. 2-(4-((4-benzamidophenyl)amino)-1H-pyrazolo[3,4-d]pyrimidin-1-yl)acetic acid (**6d**)*

61%; White solid; mp > 240 □; ^{1}H NMR (400 MHz, DMSO-d_6) *δ*: 13.20 (br, 1H, COOH), 10.30 (br, 1H, NH), 10.12 (br, 1H, NH), 8.41 (s, 1H, CH), 8.25 (s, 1H, CH), 7.97 (d, *J* = 7.74 Hz, 2H, ArH), 7.76–7.83 (m, 4H, ArH), 7.52–7.62 (m, 3H, ArH), 5.13 (s, 2H, CH_2); ^{13}C NMR (100 MHz, DMSO-d_6) 169.70, 165.84, 156.00, 154.88, 154.02, 135.44, 135.16, 133.06, 132.69, 131.96, 128.84, 128.09, 122.28, 121.27, 101.29, 48.50; IR (KBr, ν, cm^{-1}): 3306, 3113, 2922, 1738, 1633, 1530, 1491, 1405, 1300,1266, 1216, 1042, 926, 815, 717, 678, 543, 416. HRMS (ESI) calcd for $C_{20}H_{16}N_6O_3$ $[M + H]^+$, 388.1284; found, 389.1367.

*4.1.16. 2-(4-((4-(2-chloroacetamido)phenyl)amino)-1H-pyrazolo[3,4-d]pyrimidin-1-yl)acetic acid (**6e**)*

64%; Pink solid; mp > 240 □; ^{1}H NMR (400 MHz, DMSO-d_6) *δ*: 13.19 (br, 1H, COOH), 10.35 (br, 1H, NH), 10.13 (br, 1H, NH), 8.40 (s, 1H, CH), 8.24 (s, 1H, CH), 7.78 (d, *J* = 8.60 Hz, 2H, ArH), 7.63 (d, *J* = 8.96 Hz, 2H, ArH), 5.12 (s, 2H, CH_2), 4.27 (s, 2H, CH_2); ^{13}C NMR (100 MHz, DMSO-d_6) 169.67, 164.89, 155.89, 154.74, 153.96, 135.29, 134.82, 132.72, 122.48, 120.27, 101.29, 48.49, 44.04; IR (KBr, *v*, cm^{-1}): 3296, 3126, 3072, 2924, 2854, 2507, 1914, 1652, 1607, 1556, 1510, 1464, 1414, 1380, 1275, 1255, 1219, 1144, 1041, 969, 840, 775, 731, 700, 642, 619, 520; HRMS (ESI) calcd for $C_{15}H_{13}ClN_6O_3$ $[M + H]^+$, 360.0738; found, 361.0823.

*4.1.17. 2-(4-((4-acetamidophenyl)amino)-1H-pyrazolo[3,4-d]pyrimidin-1-yl)acetic acid (**6f**)*

58%; Pink solid; mp > 240 □; ^{1}H NMR (400 MHz, DMSO-d_6) *δ*: 13.20 (br, 1H, COOH), 10.20 (br, 1H, NH), 10.01 (br, 1H, NH), 8.26–8.43 (m, 2H, CH), 7.60–7.72 (m, 4H, ArH), 5.12 (s, 2H, CH_2), 2.05 (s, 3H, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6) 169.13, 168.06, 155.15, 153.36, 133.76, 132.35, 119.34, 100.69, 48.02, 23.90; IR (KBr, *v*, cm^{-1}): 3296, 2923, 1666, 1613, 1558, 1512, 1382, 1277, 1220, 1093, 1047, 968, 837, 781, 702, 619; HRMS (ESI) calcd for $C_{15}H_{14}N_6O_3$ $[M + H]^+$, 326.1127;

found, 327.1141.

4.2 Determination of XOD inhibitory activity

Measurement of XOD inhibition was carried out by slightly modifying the method of Cos et al [23-25]. First, 1092 μL of 0.1 unit of XOD in buffer (200 mM sodium pyrophosphate/HCl, pH 7.5) and 2 μL(5, 10, 15, and 25 μM) of the test extracts or compounds in DMSO were mixed at 37 □ for 5 min. The control group did not contain the test agent. The reaction started by adding 200 μL of 0.6 mM xanthine in double distilled water to the mixture. The reaction mixture was incubated at ambient temperature. Finally, absorption increments at 295 nm indicating the formation of uric acid were determined every minute up to 8 min. Allopurinol was used as the positive control. Three replicates were made for each test sample. The inhibition ratio (%) was calculated according to the equation [(rate of control reaction − rate of sample reaction)/rate of control reaction] × 100.

4.3 Antitumour activity assay

4.3.1. Cells and culture.

The human hepatoma carcinoma cell lines 7402 and 7221 were obtained from the State Key Laboratory of Biotherapy, Sichuan University. Logarithmically growing human hepatoma carcinoma cells 7402 and 7221 were incubated with 0.05% trypsin containing 1 mM EDTA at 37 °C for about 4 min until the cells were nonadherent and formed a single-cell suspension. Trypsin activity was neutralized by adding a 20-fold excess of serum-containing medium. The cells were cultured at 37 ℃ in air with 5% CO_2.

4.3.2. Cell survival assay.

Cell viability was assayed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Briefly, human hepatoma carcinoma cells 7402 and 7221 were plated at a density of 1×10^5 cells/mL into 96-well plates and 7×10^3-well plates, respectively. After overnight growth, the cells were pretreated with a series of concentrations of acacetin for 24 h. The final concentrations of dimethyl sulfoxide in the culture medium were <0.1%. At the end of the treatment, 10 μL of MTT was added, and the cells were further incubated for 4 h. Cell viability was determined by scanning with an ELISA reader using a 570 nm filter [26-28].

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References

[1] E. Honkanen, A. Pippuri, P. Kairisalo, P. Nore, H. Karppanen, I. Paakkari, J. Med.Chem. 26 (10) (1983) 1433-1438.

[2] N. M. Abdel Gawad, H. H. Georgey, R. M. Youssef, N. A. El-Sayed, Eur. J. Med. Chem. 45(2010) 6058-6067.

[3] M. N. Noolvi, H. M. Patel, V. Bhardwaj, A. Chauhan, Eur. J. Med. Chem. 46 (2011) 2327-2346.

[4] S. M. El-Messery, G. S. Hassan, F. A. M. Al-Omary, H. I. El-Subbagh, Eur. J. Med. Chem.

54 (2012) 615-625.

[5] P. Mirjana, S. Sasa, S. Milos, K. Vesna, B. Gordana, P. Velimir, Bioorg. Med. Chem. Lett. 22(2012) 6700-6704.

[6] T. S. F. Md, Z. Noha I., S. Fabio, F. Stefano, W. Andrew D, Eur. J. Med. Chem. 56(2012) 263-270.

[7] D. C. Kim, Y. R. Lee, B.-S. Yang, K. J. Shin, D. J. Kim, B. Y. Chung, K. H. Yoo, Eur. J. Med. Chem. 38(2003) 525-532.

[8] O. Moukha-Chafiq, M. L. Taha, H. B. Lazrek, C. Pannecouque, M. Witvrouw, E. De lercq, J. L. Barascut, J. L. Imbach, NUCLEOSIDES, NUCLEOTIDES & NUCLEIC ACIDS. 20(10&11)(2001) 1797-1810.

[9] H. Tamta, S. Kalra, A. K. Mukhopadhyay, Biochemistry. 71(2006) 49-54.

[10] S. Gupta, L. M. Rodrigues, A. P. Esteves, Eur. J. Med. Chem. 43(4) (2008) 771-780.

[11] H. S. D. Naeff, M. C. R. Franssen, H. C. vander Plas, Recl. Trav. Chim. Pays-Bas. 110(5) (2001) 139-150.

[12] S. Kalra, G. Jena, K. Tikoo, A. K Mukhopadhyay, BMC Biochem, 8 (2007) 8.

[13] B. Alberto, M. Loana, D. Sabrina, M. Lucio, G. Grazia, C. Andrea, G. Giuseppe, C. Walter, P. Sergio, V. Loredana, C. Massimo, M. F. Maria, C. M. Luisa, B. Marcella, P. Claudio, G. Chiara, Z. Valentina, D. C. Michelandrea, Z. Franco, Eur. J. Med. Chem. 53(2012) 64-75.

[14] D. Roumyana, G. Galina, G. Konstantin, CR Acad. Bulg. Sci. 65(6) (2012) 815-820.

[15] J. Ge, E. Normant, J. R. Porter, J. A. Ali, M. S. Dembski, Y. Gao, A. T. Georges, L. Grenier, R.H. Pak, J. Patterson, J.R. Sydor, T. T. Tibbitts, J. K. Tong, J. Adams, V. J. Palombella, J. Med. Chem. 49(2006) 4606-4615.

[16] G. Chiosis , B. Lucas , A. Shtil , H. Huezo , N. Rosen, Bioorg. Med. Chem. 10(2002) 3555.

[17] M. S. Mohamed, A. E. Rashad, M. E. Zaki, S. S. Fatahala, Acta. Pharm. 55(2005) 237-249.

[18] A. Davoodnia, R. Zhiani, N. Tavakoli-Hoseini, Monatsh Chem. 139(2008) 1405-1407.

[19] S.-M. Yang, R. H Scannevin, W. V Murray, Bioorg. Med. Chem. Lett. 8(2008) 1135–1139.

[20] Y. Wang, S. Frank, Jr. Guziec, J. Org. Chem. 66(2001) 8293-8296.

[21] M. L. Bolognesi, A. Cavalli, L.Valgimigli, M. Bartolini, M. Rosini,V. Andrisano, M. Recanatini, C. Melchiorre, J. Med. Chem. 50(2007) 6446-6449.

[22] L. Keurulainen, O. Salin, A. Siiskonen, J. M. Kern, J. Alvesalo, P. Kiuru, M. Maass, J. Yli-Kauhaluoma, P. Vuorela, J. Med. Chem. 53(2010)7664-7674.

[23] P. Cos, L. Ying, M. Calomme, J. Nat. Prod. 61(1998) 71-76.

[24] C. Enroth, B. T. Eger, K. Okamoto, T. Nishino, E. F. Pai, Proc. Natl. Acad. Sci. U.S.A. 97(2000) 10723-10728.

[25] T. Ardan, J. Kovaceva, J. Cejkova, Acta Histochem. 106(2004) 69-75.

[26] F. Caruso, M. Rossi, J. Tanski, C. Pettinari, F. Marchetti, J. Med. Chem. 46(2003) 1737-1742.

[27] N. M. O'Boyle, M. Carr, L. M. Greene, O. Bergin, S. M. Nathwani, T. McCabe, D. G. Lloyd, D. M Zisterer, M. J. Meegan, J. Med. Chem. 53(2010) 8569-8584.

[28] P. Franchetti, L. Cappellacci, M. Pasqualini, R. Petrelli, P. Vita, H. N. Jayaram, Z. Horvath, T. Szekeres, M. Grifantini, J. Med. Chem. 48(2005) 4983-4989.

Tables

Table 1. XOD inhibitory activity of new compounds [a]

compounds	IC_{50}/μg·mL^{-1}
allopurinol	1.60
3	inactive
4	86.65
5a	inactive
5b	>100
5c	77.74
5d	34.10
5e	40.59
5f	38.79
6a	>100
6b	89.28
6c	61.25
6d	40.32
6e	36.83
6f	33.77

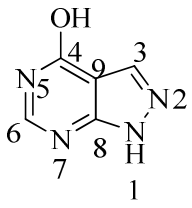
[a] The IC_{50} value (μg·mL^{-1}) of the reducing power assay is the effective concentration at which the absorbance is 0.5 of the reducing power.

Table 2. In vitro activities of compounds (IC_{50} in μM) against human hepatoma carcinoma cells 7402 and 7221[a]

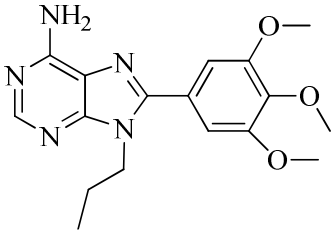
compounds	IC_{50}($\mu g \cdot mL^{-1}$) 72h	
	7402	7221
17-AAG	7.27	5.77
Allopurinol	--	--
3	>100	>100
4	>64	>64
5a	>64	>64
5b	>64	>64
5c	>100	>100
5d	>100	>100
5e	7.27	3.51
5f	>64	>64
6a	>64	>64
6b	>100	>100
6c	>100	>100
6d	>100	>100
6e	>100	>100
6f	>64	>64

[a] IC_{50} values represent the drug concentrations required to inhibit cancer cell replication by 50%. The compounds were tested up to a concentration of 64 μM. IC_{50} values were calculated by probit analysis ($P < 0.05$, χ^2 test).

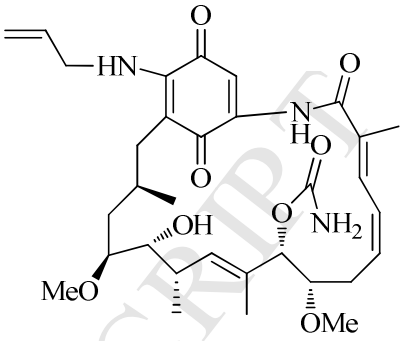
Figures



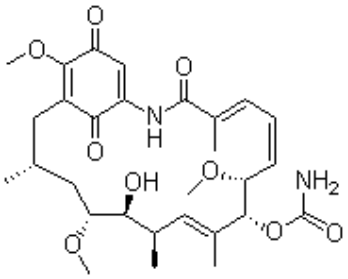
allopurinol



PU$_3$

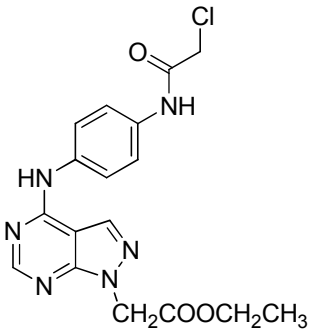


17AAG



Geldanamycin

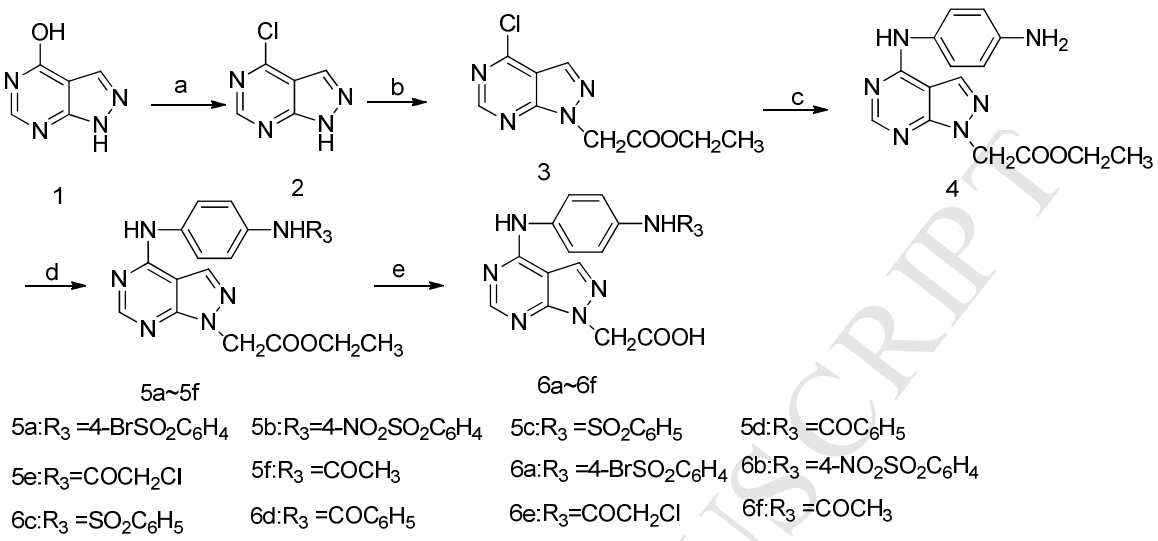
Fig. 1 Compound allopurinol, **PU**3, natural product Hsp90 inhibitor (**17AAG**) and geldanamycin



5e

Fig. 2 Structures of the active antitumour agents.

Schemes



Scheme 1. Reagents and conditions: (a) $POCl_3$, $(CH_3)_2NC_6H_5$, 80 °C; (b) $BrCH_2COOCH_2CH_3$, TEA, DMF, rt; (c) $H_2NC_6H_4NH_2$, CH_3CN, 80 °C; (d) NaH, THF, 0 °C, Sulfonyl chloride or Chloride; (e) NaOH (1N), CH_3CH_2OH, rt.

Highlights

►A series of pyrazolo[3,4-d]pyrimidines analogues were designed and synthesized.

► *In-vitro* Xanthine Oxidase inhibitory activity and antitumor activity of the new compounds were evaluated.

►Compound **5e** possessed the best antitumor activity than 17AAG and allopurinol.