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Preclinical report

Synthesis and anticancer activities of 4-oxobenzopyrano[2,3-*d*]pyrimidines

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Several 2-aryl-4-oxoxbenzopyrano[2,3-*d*]pyrimidines have previously been shown to exhibit *in vivo* antitumor activity in mice with P388 lymphocytic leukemia. In the present study, a series of novel substituted benzopyrano[2,3-*d*]pyrimidines have been prepared and tested for cytotoxic activity against a panel of cancer cell lines including the P388 lymphocytic leukemia cell line. The unsubstituted parent compound, some methoxylated derivatives and a cyclohexyl derivative all exhibited potent cytotoxic activity (IC_{50} values 0.3–0.64 μM). A number of derivatives, including the unsubstituted parent pyrimidine, were shown to cause a significant perturbation in cell cycle kinetics with an observed 2- to 3-fold increase in cells in the G_2/M phase of the cell cycle. Furthermore, a polymethoxylated derivative, 2-(3,4,5-trimethoxyphenyl)-9-methoxy-4-oxo-2,3-dihydrobenzopyrano[2,3-*d*]pyrimidine 13, was shown to be selectively active against a number of human ovarian cell lines. [© 1999 Lippincott Williams & Wilkins.]

Key words: Antitumor, ovarian cells, pyrimidines.

Introduction

Several 2-aryl-4-oxobenzopyrano [2,3-*d*] pyrimidines have previously been shown to exhibit significant *in vivo* antitumor activities.[1,2] For example, the parent phenyl compound (**1**) was able to increase the mean survival time of mice with P388 lymphocytic leukemia by 65% relative to controls at a dose of 128 mg/kg. In addition, a 2-(4-tolyl) substituted derivative gave an even higher mean survival time of 86% for a dose of 256 mg/kg. The potent *in vivo* activities displayed by this class of compound led us to further evaluate their biological potential,[3,4] and herein we describe the synthesis and biological activities of a range of novel substituted benzopyrano[2,3-*d*]pyrimidines. Compounds were tested for *in vitro* cytotoxicity against a panel of cancer cell lines, including the P388 lymphocytic leukemia cell line, and for their ability to alter cell cycle kinetics. See Figure 1.

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Materials and methods

Chemicals

Melting points (m.p.) were recorded on a Leica Galen III hot-stage melting point apparatus and are uncorrected. NMR spectra were recorded at 250 MHz on a Bruker AC250 spectrometer in either $CDCl_3$ or DMSO solution at 303 ± 1 K using Me_4Si as internal standard. Mass spectra were determined either as electron impact mass spectra on a VG Trio-3 mass spectrometer at an ionization energy of 70 eV or on a Finnigan TSQ700 triple quadrupole mass spectrometer (San Jose, CA) operated in ESI mode with an electrode voltage of -4.5 kV. Elemental analyses were carried out by Medac (Brunel Science Center, Egham, UK). All synthetic intermediates were purchased from Aldrich (Gillingham, UK). The intermediate iminochromens were prepared from the appropriately substituted salicylaldehydes as described previously.[2]

3-Carbamoyl-2-iminochromen 4. General procedure for the preparation of iminochromens. To a mixture of salicylaldehyde (6.1 g, 50 mmol) and cyanoacetamide (4.2 g, 50 mmol) in ethanol (50 ml) was added

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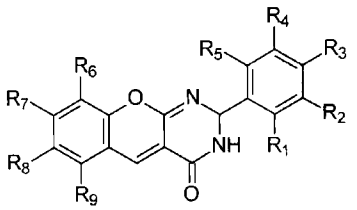
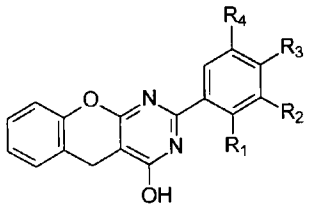
piperidine (0.5 ml) and the mixture stirred for 2 h. After standing overnight the mixture was cooled to −10°C and the crude solid that separated was collected by filtration. Recrystallization from ethanol to gave the iminochromen **4** as a cream solid (7.5 g, 80%): m.p. 189°C; *m/z* 188 (M^+, 76%), 171 (100), 145 (88), 118 (58), 89 (58), 81 (25), 69 (36) and 43 (42); δ_H ($CDCl_3$) 9.99 (1H, br s, NH), 8.48 (1H, s, H-4), 7.60 (1H, br s, NH), 7.48 (2H, m, ArH), 7.18 (2H, m, ArH) and 5.96 (1H, br s, NH); δ_C ($CDCl_3$) 164.0, 157.35, 154.0, 142.3, 132.7, 129.5, 124.0, 120.35, 118.7 and 115.2.

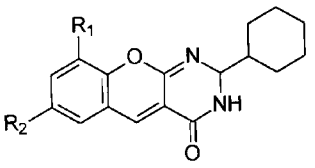
2-Phenyl-4-oxo-2, 3-dihydrobenzopyrano [2, 3-d] pyrimidine **1**. *General procedure for the preparation of benzopyrano[2,3-d]pyrimidines.* To a mixture of 3-carbamoyl-2-iminochromen **4** (1.88 g, 0.01 mol) and benzaldehyde (1.0 ml, 0.01 mol) in ethanol (100 ml) was added piperidine (0.3 ml) and the mixture heated under refluxed for 3 h. The mixture was allowed to cool and left to stand overnight. The crude product that separated was filtered off and air dried. Repeated extraction with boiling ethanol and successive recrystallization from ethanol afforded the pure pyrimidine **1** (0.8 g, 29%): m.p. 239°C; *m/z* 276 (M^+, 28%), 199 (23)



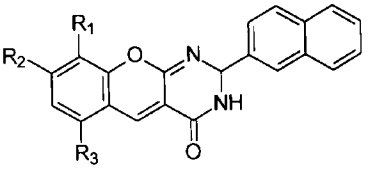
Compound	Substituents								
	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8	R_9
1	H	H	H	H	H	H	H	H	H
7	OMe	H	H	H	H	H	H	H	H
8	H	OMe	H	H	H	H	H	H	H
9	H	H	OMe	H	H	H	H	H	H
10	H	H	NO_2	H	H	H	H	OMe	H
11	H	H	H	H	Me	OMe	H	H	H
12	H	H	H	H	Me	H	H	OMe	H
13	H	OMe	OMe	OMe	H	OMe	H	H	H
14	H	H	H	H	H	OMe	H	H	H
15	H	OMe	OMe	OMe	H	H	H	H	H
16	Me	H	H	H	H	H	H	H	H
17	H	OMe	H	OMe	H	H	H	H	H
18	H	H	NO_2	H	H	H	H	H	H
19	Cl	H	H	H	H	H	H	H	H
25	OH	OMe	H	H	H	H	H	Cl	H
27	H	OMe	OMe	OMe	H	H	OMe	H	OMe
31	OH	H	H	H	H	OMe	H	H	H



20 $R_1 = R_3 = OMe$; $R_2 = R_4 = H$
21 $R_1 = R_3 = R_4 = H$; $R_2 = OMe$
22 $R_1 = R_3 = H$; $R_2 = R_4 = OMe$
23 $R_1 = OMe$; $R_2 = R_3 = R_4 = H$
24 $R_1 = H$; $R_2 = R_3 = R_4 = OMe$



26 $R_1 = H$; $R_2 = Cl$
29 $R_1 = OMe$; $R_2 = H$



28 $R_1 = H$; $R_2 = R_3 = OMe$
30 $R_1 = OMe$; $R_2 = R_3 = H$

Figure 1. Structures of 4-oxobenzopyrano[2,3-*d*]pyrimidines.

and 44 (100); δ_H (DMSO) 9.00 (1H, br s, NH), 8.03 (1H, s, H-5), 7.68 (1H, dd, J=7.6, 1.5, ArH), 7.51 (1H, dt, J=7.6, 1.5, ArH), 7.38 (5H, m, ArH), 7.21 (2H, t, J=7.6, ArH) and 6.25 (1H, t, J=1.5, H-2); δ_C (DMSO) 158.9, 154.3, 153.5, 142.3, 133.2, 133.05, 129.8, 128.6, 128.1, 126.35, 124.1, 119.2, 115.7, 114.7 and 71.1. (Found: C, 73.86; H, 4.34; N, 10.17%. Calculated for $C_{17}H_{12}N_2O_2$: C, 73.9; H, 4.38; N, 10.14.)

2-(3, 4, 5-Trimethoxyphenyl)-9-methoxy-4-oxo-2,3-dihydrobenzopyrano[2,3-d]pyrimidine ***13***. This was prepared according to the general procedure from 3-carbamoyl-2-imino-8-methoxychromen (2.19 g, 0.01 mol) and 3,4,5-trimethoxybenzaldehyde (1.96 g, 0.01 mol) as an amorphous cream solid (1.43 g, 36%): m.p. 316°C; *m/z* 397 ($[M+H]^+$, 100%), 229 (44), 220 (100), 202 (56) and 196 (45); δ_H (DMSO) 8.91 (1H, br s, NH), 7.97 (1H, s, H-5), 7.25–7.15 (3H, m, ArH), 6.68 (2H, s, H-2′ and H-6′), 6.19 (1H, s, H-2), 3.86 (3H, s, OCH_3), 3.77 (6H, s, OCH_3) and 3.65 (3H, s, OCH_3).

Cytotoxicity assay

The synthesized agents were initially tested for cytotoxic activity as dimethyl sulfoxide (DMSO) solutions in murine P388 lymphocytic leukemia, K562 human chronic myelogenous leukaemia and A2780 human ovarian cell lines. The dimethyl-thiazol-diphenyltetrazolium bromide (MTT) assay[5] was used to measure drug-induced cytostasis and IC_{50} values (concentration required to inhibit cell growth by 50% as determined by the MTT assay) are reported in Table 1. Compound **13** was further evaluated for cytotoxic behavior against a panel of cell lines including Ovca5 and Ovca433 ovarian cell lines, non-ovarian A549 and H460 cell lines, and estrogen-positive and -negative (MCF7 and BT20, respectively) human breast carcinoma cell lines (Table 2).

Cell cycle analysis

The ability of the agents to affect cell cycle kinetics (in K562 cells) was measured by flow cytometry as described previously.[6] Briefly, cells were treated with drug (10 μg/ml), followed by fixation in cold acetone:ethanol (50:50, 4°C) after 1, 2, 3, 4, 5, 6, 7, 12 and 26 h. The cells were rehydrated and washed in phosphate-buffered saline (pH 7.0) prior to staining with propidium iodide (50 μg/ml). DNA content was measured by flow cytometry (Coulter Epics V) using an argon laser (λ_{ex}=488 nm, $\lambda_{em} \geqslant 500$ nm). Coefficients of variation were less than 6% and the fraction of cells in each phase of the cell cycle was calculated using the commercial software provided by the manufacturer.

Table 1. IC_{50}s (μM) of agents **1** and **7–31** in K562 human chronic myelogenous leukemia, P388 murine lymphocytic leukemia and A2780 human ovarian cell lines

Compound	K562	P388	A2780
1	1.55	0.3	NT
7	25.1	10.3	NT
8	2.2	0.35	NT
9	1.9	2.3	NT
10	>50	>50	24.9
11	I	I	I
12	15.45	2.35	11.8
13	>10	>10	0.45
14	>50	>50	21.0
15	15.6	>50	15.4
16	7.05	0.54	6.35
17	2.64	0.48	3.05
18	>25	NT	18.7
19	14	1.1	19.5
20	15	>16	6.3
21	I	I	I
22	0.48	>10	>10
23	>12.5	>12.5	>12.5
24	I	I	I
25	>1	>1	>1
26	0.23	0.64	4.0
27	>5	>5	>5
28	0.94	1.5	1.0
29	>5	>5	4.5
30	>5	>5	>5
31	>50	>50	2.5

NT, not tested; I, insoluble.

Table 2. IC_{50}s (μM) of pyrimidine (**13**) in ovarian and non-ovarian cell lines

Ovarian cell lines			Non-ovarian cell lines	
A2780	Ovca5	Ovca433	A549	H460
0.45	5.52	2.85	>10	>10

Results and discussion

The heterocycles (**1** and **7–31**) were synthesized[1,2] according to Scheme 1. Briefly, treatment of an *o*-hydroxybenzaldehyde **2** with cyanoacetamide **3** afforded the appropriately substituted 3-carbamoyl-2-iminochromens **4** in good yields (about 80%). Reaction of the iminochromens **4** with a benzaldehyde or cyclohexanecarboxaldehyde in the presence of base

gave the desired 2-substituted-4-oxobenzopyrano[2,3-*d*]pyrimidines **5** in moderate yields. However, prolonged treatment of pyrimidines **5** under basic conditions resulted in formation[7] of the isomeric pyrimidinols **6**.

The agents were evaluated for *in vitro* cytotoxicity against a panel of tumor-derived cell lines (K562, P388 and A2780) and the results are presented in Table 1 as the concentrations required to inhibit cell growth by 50% (IC_{50} values). Against the K562 cell line, only the dimethoxy 4-hydroxy isomer (**22**), and the cyclohexyl and naphthyl-4-oxo isomers (**26** and **28**, respectively) exhibited greater activity ($IC_{50} < 1$ μM) than the unsubstituted parent pyrimidine **1**. However, in the murine P388 cell line, the unsubstituted pyrimidine **1** was the most potent agent tested (IC_{50}=0.3 μM) whilst the methoxylated (**8** and **17**), methyl (**16**) and cyclohexyl (**26**) substituted compounds all exhibited similar levels of cytotoxic activity (IC_{50}=0.35-0.64 μM).

The agents were tested for their ability to cause an alteration in the cell cycle distribution of K562 cells. Only the parent compound (**1**) and three methoxylated derivatives (**8**, **9** and **23**) were able to significantly block cells in the G_2/M phase of the cell cycle with a 2- to 3-fold increase relative to untreated controls (data not shown). The lack of uniform activity in the cell cycle across the series of compounds tested suggests that anti-mitotic action is an unlikely cause of cytotoxicity with these agents and, hence, further tubulin-based experiments were not carried out.

Apart from the polymethoxylated pyrimidine (**13**), none of the agents examined were particularly cytotoxic towards the A2780 human ovarian cell line. Indeed, this agent (**13**) was considerably more active in the ovarian cell line (IC_{50}=0.45 μM) than in the leukemic cell lines ($IC_{50} > 10$ μM). These differences in cytotoxicity led us to further examine the cytotoxic behavior of the pyrimidine **13** in other ovarian and non-ovarian tumor cell lines (Table 2). Consistent with this initial finding, agent **13** selectively exhibited greater cytotoxicity to human ovarian cell lines (A2780 carcinoma, Ovca5 adenocarcinoma and Ovca433 carcinoma) than to non-ovarian human cell lines (A549 lung carcinoma and H460 large cell lung carcinoma). In an attempt to discover whether this selective cytotoxic activity towards ovarian cell lines was hormonally derived, the pyrimidine (**13**) was tested in estrogen-negative BT20 and estrogen-positive MCF7 human breast carcinoma cell lines. However, agent **13** displayed no cytotoxic effects towards either of these cell lines ($IC_{50} > 10$ μM), thus indicating that the cytotoxicity is unlikely to be mediated via a hormonally derived mechanism.

Conclusion

Many of the pyrimidines investigated herein have shown strong cytotoxic activity towards the K562, P388 and A2780 cell lines examined. In addition, a small number of derivatives including the parent pyrimidine **1** displayed the ability to block cells in the G_2/M phase of the cell cycle. Notably, a particularly surprising finding of this study is the selective cytotoxicity exhibited by the polymethoxylated pyrimidine **13** towards ovarian cancer cell lines. Further studies on this agent (**13**) are in progress.

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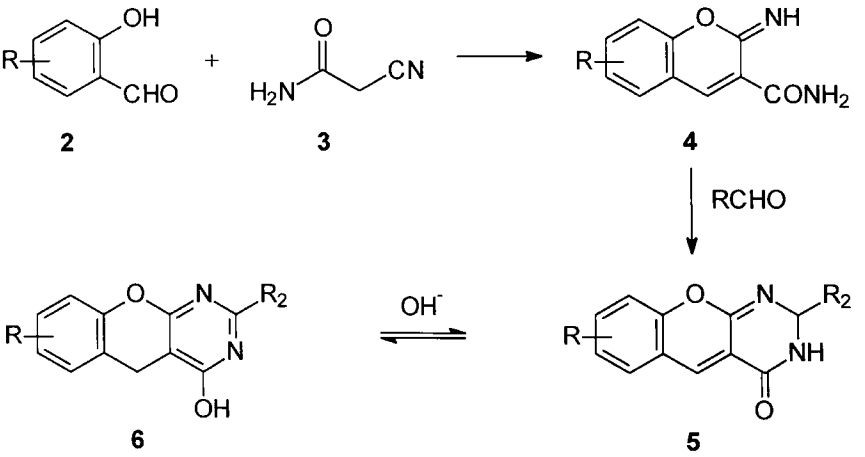

2 3 4 6 5

Scheme 1. Synthetic route to 4-oxobenzopyrano[2,3-*d*][pyrimidines.

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