JPP Journal of Pharmacy and Pharmacology

JPP 2009, 61: 1057–1066

Received November 25, 2008
Accepted May 18, 2009
DOI 10.1211/jpp/61.08.0010
ISSN 0022-3573

Research Paper

Synthesis and in-vitro antitumour activity of new naphthyridine derivatives on human pancreatic cancer cells

Irene Banti[a], Susanna Nencetti[a], Elisabetta Orlandini[a], Annalina Lapucci[a], Maria Cristina Breschi[b] and Stefano Fogli[b]

Departments of [a]Pharmaceutical Sciences and [b]Psychiatry, Neurobiology, Pharmacology and Biotechnologies, University of Pisa, Italy

Abstract

Objectives The aim of the study was to evaluate the antitumour effect *in vitro* of newly synthesized 7-substituted 2,3-dihydro-1,8-naphthyridines.

Methods Characterization tools included cell viability assay, caspase 3/7 induction, DNA fragmentation, fibroblast growth factor type 1 receptor kinase inhibition, and in-vitro antiangiogenic analysis.

Key findings Treatment of MIA PaCa-2 human pancreatic cancer cells with test compounds showed time- and concentration-dependent cytotoxicity with IC50 values in the micromolar range. Compounds with an aminoalkyl or a diaminoalkyl side chain at the 7-position exhibited remarkable cytotoxicity, whereas the presence of a methyl group or a cyclic amine in the same position led to a significant decrease in their biological activity. Cytotoxicity screening demonstrated that the most active was compound **11** (mean 50% inhibition of cell proliferation (IC50) 11 μM). This compound had an in-vitro antitumour efficacy superior to 5-fluorouracil (the lowest cell viability value after treatment (E_{max}) 0.2% and 19%, respectively) and proved to be less toxic than 5-fluorouracil against non-cancerous human oral epithelial cells. In addition, compound **11** induced apoptosis in MIA PaCa-2 cells and it was able to promote antiangiogenic effects *in vitro*. Finally, its cytotoxicity was enhanced in pancreatic cancer cells stimulated with fibroblast growth factor, while no substantial effect was observed on human bronchial smooth muscle cells stimulated with the same growth factor.

Conclusions These findings suggest that 1,8-naphthyridine derivatives are a promising class of compounds in cancer research. In particular, the antitumour activity of compound **11** is worth further investigation.

Keywords angiogenesis; apoptosis; cytotoxicity; growth factors; naphthyridines; structure–activity relationship

Introduction

Pancreatic cancer is the fourth leading cause of cancer-related deaths in western countries. Only 15–20% of patients receiving an initial diagnosis of pancreatic cancer are suitable for surgery, while the median survival time of those with metastatic disease remains short regardless of the type of therapeutic strategy employed.[1] As a matter of fact, development of resistance mechanisms for apoptosis confers high survival ability and low drug sensitivity to pancreatic tumour cells, a phenotype that has been related to the presence of a high level of anti-apoptotic proteins including survivin and XIAP, two members of the inhibitor of apoptosis (IAP) protein family. MIA PaCa-2, a poorly differentiated human pancreatic cancer cell line of ductal origin, which expressed high levels of IAPs, has shown a relatively low sensitivity to apoptosis induction by overexpression of caspase genes.[2] Attempts to increase drug efficacy by modulating pharmacokinetic parameters or by using combination schedules including different chemotherapeutic drugs and biological agents result in limited improvement,[3] demonstrating the need for novel drug development in pancreatic cancer therapy.

Naphthyridine derivatives have attracted considerable attention because the 1,8-naphthyridine skeleton is present in many compounds isolated from natural substances and exhibits various biological activities.[4] This class of compounds has also been investigated

Correspondence: Annalina Lapucci, Department of Pharmaceutical Sciences, University of Pisa, Via Bonanno 6, 56126 Pisa, Italy. E-mail: alapucci@farm.unipi.it

as potential antitumour agents and several molecules are now in different phases of clinical trials.[5] Chemical modifications of the 1,8-naphthyridine ring, including the conversion into other similar ring systems, have been reported to enhance antitumour activity of these compounds.[6,7]

On the basis of these earlier findings, we synthesized a new class of 2,3-dihydro-1,8-naphthyridine oxime ether derivatives (Table 1) as potential antiproliferative agents and tested them by means of cytotoxicity screening on MIA PaCa-2 cells. The most active compound resulting from this selection was then compared to 5-fluorouracil and characterized for its potential proapoptotic and antiangiogenic properties *in vitro* as well as for its ability to inhibit growth-stimulated cell proliferation and growth factor receptor kinase activity.

Materials and Methods

Synthesis

Melting points were determined on a Köfler hot-stage apparatus and are uncorrected. ^{1}H NMR spectra were recorded with a Varian Gemini-200 spectrometer (Varian, Palo Alto, CA, USA) operating at 200 MHz, in approximately 2% $CDCl_3$ solution.

Mass spectra were detected with a Hewlett Packard 5988A spectrometer (Hewlett Packard, Mississauga, Canada) (EI, 70 eV). Analytical thin-layer chromatography (TLC) was carried out using 0.25 mm layer silica gel plates containing a fluorescent indicator; spots were detected under UV light (254 nm). Preparative TLC was carried out on 1 or 2 mm layer silica gel plates containing a fluorescent indicator; spots were detected under UV light (254 nm). Column chromatography was performed using 70–230 mesh silica gel. Evaporation was performed *in vacuo* (rotating evaporator). Na_2SO_4 was used as the drying agent. Elemental analyses were performed by our analytical laboratory and agreed with theoretical values to within ± 0.4%.

General procedure for the synthesis of 7-substitued (E)*-hydroxyimino-2,3-dihydro-1,8-naphthyridine*

Refer to Figure 1.

A solution of 2.19 mmol of the oxime (**17**) in 67.28 mmol of the appropiate amine was stirred in a sealed tube at 120°C for 72 h. The resulting solution was concentrated, diluted with H_2O and extracted with $CHCl_3$. The organic phase was filtered and evaporated to afford a brown solid that was purified by flash chromatography on silica gel, eluting with EtOAc/hexane in adequate proportions (7:3) to give the oximes **19–25**.

19 was prepared using propylamine; purification by flash chromatography (FC) with EtOAc/hexane (7 : 3), white solid (55% yield); m.p. 180–182°C; ^{1}H NMR ($CDCl_3$) δ 8.01 (s, 1H), 7.71–7.67 (d, J = 8.6 Hz, 1H), 5.88–5.83 (d, J = 8.6 Hz, 1H), 3.33–3.20 (m, 4H), 2.74 (t, J = 6.5 Hz, 2H), 1.63–1.52 (m, 2H), 0.92 (t, J = 7.3 Hz, 3H); MS (EI, 70 eV) *m/e* 220 (M^+); Anal. $C_{11}H_{19}N_4O$ (C, H, N).

20 was prepared using butylamine; purification by FC with EtOAc/hexane (7 : 3), oil (56% yield); ^{1}H NMR ($CDCl_3$) δ 7.92–7.88 (d, J = 8.6 Hz, 1H), 5.87–5.82 (d, J = 8.6 Hz, 1H), 3.46–3.38 (m, 2H), 3.27–3.24 (m, 2H), 2.86 (t, J = 6.5 Hz, 2H), 1.61–1.39 (m, 4H), 0.94 (t, J = 7.3 Hz, 3H); MS (EI, 70 eV) *m/e* 234 (M^+); Anal. $C_{12}H_{18}N_4O$ (C, H, N).

21 was prepared using pentylamine; purification by FC with EtOAc/hexane (6 : 4), yellow solid (44% yield); m.p. 185–186°C ^{1}H NMR ($CDCl_3$) δ 7.85–7.81 (d, J = 8.6 Hz, 1H), 5.86–5.81 (d, J = 8.6 Hz, 1H), 3.42–3.35 (m, 2H), 3.27–3.18 (m, 2H), 3.05–2.98 (m, 2H), 2.86 (t, J = 6.5 Hz, 2H), 1.80–1.76 (m, 2H), 1.64–1.57 (m, 2H), 0.90 (t, J = 7.3 Hz, 3H); MS (EI, 70 eV) *m/e* 248 (M^+); Anal. $C_{13}H_{20}N_4O$ (C, H, N).

22 was prepared using *N*,*N*-diethylaminoethylamine; purification by FC with AcOEt/MeOH/TEA (9 : 1 : 0.28), oil (45% yield); ^{1}H NMR ($CDCl_3$) δ 7.78–7.74 (d, J = 8.6 Hz, 1H), 5.85–5.81 (d, J = 8.6 Hz, 1H), 3.37–3.27 (m, 4H), 2.86 (t, J = 6.5 Hz, 2H), 2.68–2.51 (m, 6H), 1.02 (t, J = 7.1 Hz, 6H); MS (EI, 70 eV) *m/e* 277 (M^+); Anal. $C_{14}H_{23}N_5O$ (C, H, N).

23 was prepared using *N*,*N*-diethylaminopropylamine; purification by FC with AcOEt/MeOH/TEA (9 : 1 : 0.28), oil (30% yield); ^{1}H NMR ($CDCl_3$) δ 7.78–7.74 (d, J = 8.6 Hz, 1H), 5.80–5.76 (d, J = 8.6 Hz, 1H), 3.37–3.24 (m, 4H), 2.85 (t, J = 6.5 Hz, 2H), 2.57–2.47 (m, 6H), 1.77–1.71 (m, 2H), 1.02 (t, J = 7.1 Hz, 6H); MS (EI, 70 eV) *m/e* 291 (M^+); Anal. $C_{15}H_{25}N_5O$ (C, H, N).

24 was prepared using piperidine; purification by FC with EtOAc/hexane (6 : 4), oil (46% yield); ^{1}H NMR ($CDCl_3$) δ 7.88–7.83 (d, J = 8.9 Hz, 1H), 6.11–6.06 (d, J = 8.9 Hz, 1H), 3.65–3.49 (m, 6H), 2.59 (t, J = 6.5 Hz, 2H), 1.61 (m, 6H); MS (EI, 70 eV) *m/e* 244 (M^+); Anal. $C_{13}H_{16}N_4O$ (C, H, N).

25 was prepared using *N*-methylpiperazine; purification by FC with EtOAc/hexane (6 : 4), solid (57% yield); mp 145–148°C; ^{1}H NMR ($CDCl_3$) δ 7.84–7.80 (d, J = 8.9 Hz, 1H), 6.06–6.01 (d, J = 8.9 Hz, 1H), 3.56–3.51 (m, 4H), 3.35–3.29 (m, 2H), 2.83 (t, J = 6.5 Hz, 2H), 2.50–2.45 (m, 4H), 2.28 (s, 3H); MS (EI, 70 eV) *m/e* 261 (M^+); Anal. $C_{13}H_{19}N_5O$ (C, H, N).

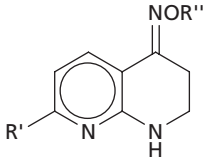
General procedure for the synthesis of 7-substitued (E)*-hydroxyimino-2,3-dihydro-1,8-naphthyridine ethers*

Refer to Figure 1.

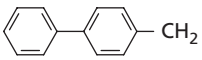
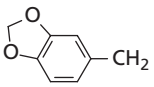
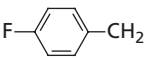
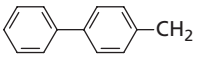
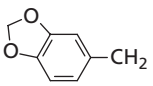
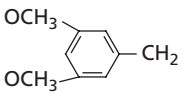
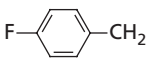
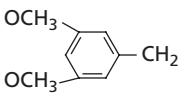
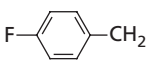
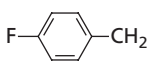
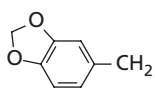
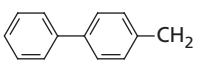
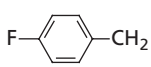
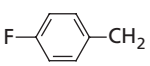
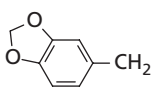
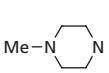
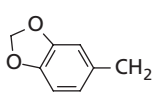
A stirred solution of 0.64 mmol of the appropriate 7-substituted oxime (**19–25**) in 5.8 ml of dry DMF was cooled to 0°C and treated portionwise with NaH (60% mineral oil dispersion, 0.082 g, 1.97 mmol). To the resulting mixture was added dropwise while stirring a solution of 2.60 mmol of appropiate arylmethylhalogenide in 2.3 ml of dry DMF. The reaction mixture was stirred at room temperature for 30 min and the hydride excess was decomposed with small amount of EtOAc. After evaporation to dryness, the oil residue was submitted to flash chromatography on silica gel, eluting with EtOAc/hexane or EtOAc/MeOH/TEA in adequate proportions to give the oxime ethers desired (**1–16**; Table 1).

1 was prepared using phenylbenzyl chloride; purification by FC with EtOAc/hexane (6 : 4); solid (51% yield); m.p. 153–155°C; ^{1}H NMR ($CDCl_3$) δ 8.00–7.96 (d, J = 7.7 Hz,

Table 1 (*E*)-oxime ethers **1–16**



1–16

Compound	R'	R''	IC50 (μM)*
1	CH_3		>50
2	CH_3		>50
3	$CH_3(CH_2)_2NH$		20 ± 1.6
4	$CH_3(CH_2)_2NH$		25 ± 1.4
5	$CH_3(CH_2)_2NH$		30 ± 1.3
6	$CH_3(CH_2)_2NH$		34 ± 1.6
7	$CH_3(CH_2)_3NH$		16 ± 1.7
8	$CH_3(CH_2)_4NH$		23 ± 1.0
9	$CH_3(CH_2)_4NH$		18 ± 2.8
10	$(Et)_2N(CH_2)_2NH$		12 ± 1.9
11	$(Et)_2N(CH_2)_3NH$		11 ± 0.5
12	$(Et)_2N(CH_2)_3NH$		12 ± 1.1
13	$(Et)_2N(CH_2)_3NH$		17 ± 1.8
14			25 ± 1.1
15			22 ± 1.1
16			25 ± 1.4

*When the IC50 was not reached at 50 μM (i.e. the maximum concentration tested), compounds were considered to be inactive. Data are mean ± SEM. of three measurements.

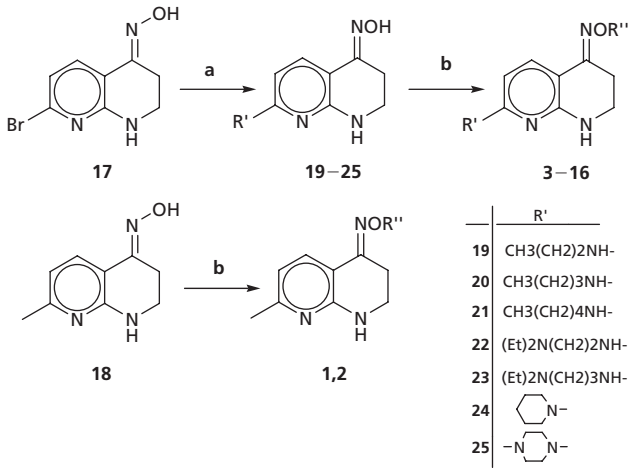


Figure 1 Synthetic route for preparing *(E)*-oxime ethers **1–16**. a, amine, 120–150°C; b, NaH (oil mineral dispersion 60%), arylmethylhalogenide, DMF anhydrous, N_2, 80°C.

1H), 7.61–7.34 (m, 9H), 6.53–6.50 (d, J = 7.7 Hz, 1H), 5.23 (s, 2H), 3.40–3.33 (m, 2H), 2.89 (t, J = 6.5 Hz, 2H), 2.37 (s, 3H); MS (EI, 70 eV) *m/e* 343 (M^+); Anal. $C_{22}H_{21}N_3O$ (C, H, N).

2 was prepared using 3,4-methylendioxybenzyl bromide;[8] purification by FC with EtOAc/hexane (7 : 3); oil (30% yield); ^{1}H NMR ($CDCl_3$) δ 7.97–7.94 (d, J = 7.8, 1H), 6.90–6.76 (m, 3H), 6.52–6.48 (d, J = 7.8 Hz, 1H), 5.95 (s, 2H), 5.06 (s, 2H), 3.38–3.30 (m, 2H), 2.84 (t, J = 6.5 Hz, 2H), 2.36 (s, 3H); MS (EI, 70 eV) *m/e* 311 (M^+); Anal. $C_{17}H_{17}N_3O_3$ (C, H, N).

3 was prepared using *p*-fluorobenzyl chloride; purification by FC with EtOAc/hexane (7 : 3); oil (38% yield); ^{1}H NMR ($CDCl_3$): δ 7.88–7.84 (d, J = 8.6 Hz, 1H); 7.41–7.34 (m, 2H); 7.08–6.99 (m, 2H); 5.84–5.80 (d, J = 8.6 Hz, 1H); 5.10 (s, 2H); 3.36–3.28 (m, 2H); 3.22–3.16 (m, 2H); 2.82 (t, J = 6.5 Hz, 2H); 1.71–1.53 (m, 2H); 0.98 (t, J = 7.3 Hz, 3H); MS (EI, 70 eV) *m/e* 328 (M^+); Anal. $C_{18}H_{21}FN_4O$ (C, H, N).

4 was prepared using phenylbenzyl chloride; purification by FC with EtOAc/hexane (7 : 3); oil (30% yield); ^{1}H NMR ($CDCl_3$): δ 7.91–7.87 (d, J = 8.6 Hz, 1H); 7.61–7.33 (m, 9H); 5.84–5.80 (d, J = 8.6 Hz, 1H); 5.18 (s, 2H); 3.37–3.29 (m, 2H); 3.25–3.15 (m, 2H); 2.86 (t, J = 6.5 Hz, 2H); 1.66–1.65 (m, 2H); 0.97 (t, J = 7.3 Hz, 3H); MS (EI, 70 eV) *m/e* 386 (M^+); Anal. $C_{24}H_{26}N_4O$ (C, H, N).

5 was prepared using 3,4-methylendioxybenzyl bromide;[8] purification by FC with EtOAc/hexane (6 : 4); oil (40% yield); ^{1}H NMR ($CDCl_3$): δ 7.87–7.83 (d, J = 8.6 Hz, 1H); 6.90–6.74 (m, 3H); 5.82–5.78 (d, J = 8.6 Hz, 1H); 5.93 (s, 2H); 5.01 (s, 2H); 3.30–3.12 (m, 4H); 2.80 (t, J = 6.5 Hz, 2H); 1.66–1.49 (m, 2H); 0.95 (t, J = 7.3 Hz, 3H); MS (EI, 70 eV) *m/e* 354 (M^+); Anal. $C_{19}H_{22}N_4O_3$ (C, H, N).

6 was prepared using 3,5-dimethoxybenzyl bromide; purification by FC with EtOAc/hexane (7 : 3); oil (40% yield); ^{1}H NMR ($CDCl_3$): δ 7.89–7.85 (d, J = 8.6 Hz, 1H); 6.57–6.40 (m, 3H); 5.84–5.80 (d, J = 8.6 Hz, 1H); 5.09 (s, 2H); 3.80 (s, 6H); 3.36–3.30 (m, 2H); 3.25–3.19 (m, 2H); 2.86 (t, J = 6.5 Hz, 2H); 1.67–1.56 (m, 2H); 0.98 (t, J = 7.3 Hz, 3H); MS (EI, 70 eV) *m/e* 370 (M^+); Anal. $C_{20}H_{26}N_4O_3$ (C, H, N).

7 was prepared using p-fluorobenzyl chloride; purification by FC with EtOAc/hexane (7 : 3); oil (35% yield); ^{1}H NMR ($CDCl_3$): δ 7.85–7.81 (d, J = 8.6 Hz, 1H); 7.39–7.32 (m, 2H); 7.06–6.97 (m, 2H); 5.82–5.78 (d, J = 8.6 Hz, 1H); 5.07 (s, 2H); 3.32–3.16 (m, 4H); 2.80 (t, J = 6.5 Hz, 2H); 1.59–1.32 (m, 4H); 0.92 (t, J = 7.3 Hz, 3H); MS (EI, 70 eV) *m/e* 342 (M^+); Anal. $C_{19}H_{23}FN_4O$ (C, H, N).

8 was prepared using 3,5-dimethoxybenzyl bromide; purification by FC with EtOAc/hexane (7 : 3); oil (33% yield); ^{1}H NMR ($CDCl_3$): δ 7.87–7.83 (d, J = 8.6 Hz, 1H); 6.55–6.50 (m, 2H); 6.39 (m, 1H); 5.82–5.77 (d, J = 8.6 Hz, 1H); 5.07 (s, 2H); 3.78 (s, 6H); 3.35–3.27 (m, 2H); 3.26–3.16 (m, 2H); 2.85 (t, J = 6.5 Hz, 2H); 1.61–1.50 (m, 2H); 1.36–1.29 (m, 4H); 0.90 (t, J = 7.3 Hz, 3H); MS (EI, 70 eV) *m/e* 398 (M^+); Anal. $C_{22}H_{30}N_4O_3$ (C, H, N).

9 was prepared using *p*-fluorobenzyl chloride; purification by FC with EtOAc/hexane (6 : 4); oil (64% yield); ^{1}H NMR ($CDCl_3$) δ 8.04–7.99 (d, J = 8.6 Hz, 1H), 7.37–7.31 (m, 2H), 7.08–6.99 (m, 2H), 5.86–5.82 (d, J = 8.6 Hz, 1H), 5.06 (s, 2H), 3.46–3.40 (m, 2H), 3.30–3.21 (m, 2H), 2.82 (t, J = 6.5 Hz, 2H), 1.65–1.59 (m, 2H), 1.36–1.32 (m, 4H), 0.90 (t, J = 7.3 Hz, 3H); MS (EI, 70 eV) *m/e* 356 (M^+); Anal. $C_{20}H_{25}FN_4O$ (C, H, N).

10 was prepared using *p*-fluorobenzyl chloride; purification by FC with EtOAc/MeOH/TEA (9 : 1 : 0.28); oil (43% yield); ^{1}H NMR ($CDCl_3$) δ 7.85–7.81 (d, J = 8.6, 1H), 7.40–7.32 (m, 2H), 7.05–6.92 (m, 2H), 5.82–5.78 (d, J = 8.6 Hz, 1H), 5.06 (s, 2H), 3.35–3.32 (m, 2H), 3.18–3.12 (m, 2H), 2.77 (t, J = 6.5 Hz, 2H), 2.62–2.48 (m, 6H), 0.99 (t, J = 7.1 Hz, 6H); MS (EI, 70 eV) *m/e* 385 (M^+); Anal. $C_{21}H_{28}FN_5O$ (C, H, N).

11 was prepared using 3,4-methylendioxybenzyl bromide;[8] purification by FC with EtOAc/MeOH/TEA (9 : 1 : 0.28); oil (30% yield); ^{1}H NMR ($CDCl_3$) δ 7.83–7.79 (d, J = 8.6 Hz, 1H), 6.91–6.73 (m, 3H), 5.82–5.78 (d, J = 8.6 Hz, 1H), 5.94 (s, 2H), 5.01 (s, 2H), 3.49–3.26 (m, 4H), 2.80 (t, J = 6.5 Hz, 2H), 2.60–2.57 (m, 6H), 1.80–1.73 (m, 2H), 1.07 (t, J = 7.1 Hz, 6H); MS (EI, 70 eV) *m/e* 425 (M^+); Anal. $C_{23}H_{31}N_5O_3$ (C, H, N).

12 was prepared using phenylbenzyl chloride; purification by FC with EtOAc/MeOH/TEA (9 : 1 : 0.28); oil (35% yield); ^{1}H NMR ($CDCl_3$) δ 7.86–7.81 (d, J = 8.6 Hz, 1H), 7.61–7.33 (m, 9H), 5.82–5.78 (d, J = 8.6 Hz, 1H), 5.17 (s, 2H), 3.39–3.30 (m, 4H), 2.85 (t, J = 6.5 Hz, 2H), 2.60–2.49 (m, 6H), 1.83–1.74 (m, 2H), 1.04 (t, J = 7.1 Hz, 6H); MS (EI, 70 eV) *m/e* 457 (M^+); Anal. $C_{28}H_{35}N_5O$ (C, H, N).

13 was prepared using *p*-fluorobenzyl chloride; purification by FC with EtOAc/MeOH/TEA (9 : 1 : 0.28); oil (35% yield); ^{1}H NMR ($CDCl_3$) δ 7.82–7.78 (d, J = 8.6 Hz, 1H), 7.40–7.33 (m, 2H), 7.06–6.98 (m, 2H), 5.82–5.77 (d, J = 8.6 Hz, 1H), 5.08 (s, 2H), 3.33–3.26 (m, 4H), 2.81 (t, J = 6.5 Hz, 2H), 2.61–2.50 (m, 6H), 1.78–1.68 (m, 2H), 1.05 (t, J = 7.1 Hz, 6H); MS (EI, 70 eV) *m/e* 399 (M^+); Anal. $C_{22}H_{30}FN_5O$ (C, H, N).

14 was prepared using *p*-fluorobenzyl chloride; purification by FC with EtOAc/hexane (6 : 4); oil (40% yield); ^{1}H NMR ($CDCl_3$) δ 7.92–7.88 (d, J = 8.9 Hz, 1H),

7.32–7.25 (m, 2H), 7.03–6.95 (m, 2H), 6.09–6.04 (d, J = 8.9 Hz, 1H), 4.79 (s, 2H), 3.65–3.60 (m, 4H), 3.40 (t, J = 7 Hz, 2H), 2.56 (t, J =7 Hz, 2H), 1.74–1.56 (m, 6H); MS (EI, 70 eV) *m/e* 354 (M^+); Anal. $C_{20}H_{23}FN_4O$ (C, H, N).

15 was prepared using 3,4-methylendioxybenzyl bromide;[8] purification by FC with $CHCl_3$/EtOAc/hexane (8 : 2 : 1); oil (45% yield); ^{1}H NMR ($CDCl_3$) δ 7.92–7.88 (d, J = 8.9 Hz, 1H), 6.84–6.76 (m, 3H), 6.09–6.04 (d, J = 8.9 Hz, 1H), 5.93 (s, 2H), 4.75 (s, 2H), 3.68–3.63 (m, 4H), 3.40 (t, J = 7 Hz, 2H), 2.56 (t, J = 7 Hz, 2H), 1.61–1.56 (m, 6H); MS (EI, 70 eV) *m/e* 380 (M^+); Anal. $C_{21}H_{24}N_4O_3$ (C, H, N).

16 was prepared using 3,4-methylendioxybenzyl bromide;[8] purification by FC with EtOAc/MeOH/TEA (9 : 1 : 0.28); oil (45% yield); ^{1}H NMR ($CDCl_3$) δ 7.90–7.85 (d, J = 8.8 Hz, 1H), 6.90–6.74 (m, 3H), 6.06–6.02 (d, J = 8.8 Hz, 1H), 5.93 (s, 2H), 5.02 (s, 2H), 3.58–3.53 (m, 4H), 3.32–3.25 (m, 2H), 2.80 (t, J = 6.5 Hz, 2H), 2.49–2.44 (m, 4H), 2.32 (s, 3H); MS (EI, 70 eV) *m/e* 395 (M^+); Anal. $C_{21}H_{25}N_5O_3$ (C, H, N).

Pharmacology

Cell lines and culture conditions

MIA PaCa-2, an established cell line of ductal origin that is derived from an undifferentiated human pancreatic adenocarcinoma (American Type Culture Collection, Manassas, VA, USA), was selected for the study because of its tendency to metastasize and its resistance to chemotherapy. Cells were maintained in Dulbecco's modified medium with L-glutamine (2 mM), supplied with 10% fetal bovine serum, 2.5% horse serum, 1% mix 1 : 1 penicillin (50 IU/ml) and streptomycin (50 μg/ml) (Sigma-Aldrich, Milano, Italy), in an atmosphere of 5% CO_2 and 95% air at 37°C.

The non-cancerous human oral epithelial cell line DOK was purchased from the European Collection of Cell Cultures (Salisbury, Wiltshire, UK). Cells were routinely grown in DMEM supplemented with 2 mM glutamine, 5 μg/ml hydrocortisone and 10% fetal bovine serum.

Human bronchial smooth muscle cells (BSMC) were purchased from Lonza (Walkersville, MD, USA). Cells were maintained exactly as recommended by the manufacturer in an optimized medium (SmGM-2 BulletKit, Lonza) containing 0.1% basic fibroblast growth factor (bFGF).

Anticancer drug screening

Cytotoxicity screening of compounds **1–16** was performed by using a kit based on the conversion of the tetrazolium salt WST-1 into a colored dye by mitochondrial dehydrogenase enzymes (Roche, Mannheim, Germany). Briefly, MIA PaCa-2 cells were seeded at 2×10^3 cells/100 μl of culture medium containing compounds **1–16** at 0.01–50 μM into each well of a 96-well microtiter plate, and incubated at 37°C for 72 h. At the end of drug exposure, 10 μl of WST-1 was added into each well; the cells were then incubated for an additional 60 min and the absorbance was measured at 450 nm using a microplate reader (Wallac 1420 VICTOR2TM, PerkinElmer). Inhibition of proliferation was assessed as the percentage reduction of UV absorbance of treated cells versus control cultures; 5-fluorouracil (Sigma, St Louis, MO, USA) was used as the reference compound.

Additional experiments were also performed by exposing human oral epithelial cells for 72 h to compound **11** or 5-fluorouracil at the maximum concentration used against MIA PaCa-2 cells (i.e. 50 μM).

Caspase activity assay

Enzyme activity was assessed using the Apo-ONE Homogeneous Caspase-3/7 assay substrate (Promega, Madison, WI, USA). Briefly, MIA PaCa-2 cells were seeded at 5×10^3/well and treated with compound **11** at 20, 50 and 100 μM. After 24 h of exposure, the Caspase-3/7 assay substrate was added and the fluorescence measured at excitation and emission wavelengths of 485 and 530 nm, respectively. Values were expressed as fluorescent signals generated in cells treated with compound **11** and those produced in untreated cells (vehicle alone).

DNA fragmentation assay

MIA PaCa-2 cells were treated with compound **11** at 20, 50 and 100 μM for 24 h. Cells harvested by trypsinization were combined with detached cells and apoptosis was assessed by the Cell Death Detection ELISA kit (Roche, Mannheim, Germany), based on the recognition of released nucleosomes after DNA internucleosomal fragmentation by a mouse monoclonal antibody directed against DNA and histones.

Growth stimulation assay

MIA PaCa-2 cells were grown for 2 days in complete cell culture medium and serum-starved for 24 h. Then, cells were stimulated for 10 h with recombinant human bFGF (BD Bioscience, Bedford, MA, USA) at 10 ng/ml in the absence or in the presence of compound **11** (20 μM) given 60 min before the addition of the growth factor. Unstimulated cells treated with the compound **11** were also assessed. At the end of the experiment, cell proliferation was measured according to the abovementioned WST-1 method.

Additional experiments were also performed on BSMC cultured for 24 h in a bFGF-containing medium by exposing cells to compound **11** at 20 μM.

Fibroblast growth factor type 1 receptor kinase assay

Evaluation of the effects of compound **11** on the activity of the human fibroblast growth factor type 1 receptor (FGFR1) kinase was performed by using a human recombinant enzyme expressed in insect cells (Cerep, Celle L'Evescault, France). Briefly, compound **11** or vehicle (control) was preincubated for 5 min at room temperature with the enzyme (1 ng) in a buffer containing 50 mM Hepes/NaOH (pH 7.4), 10 mM $MnCl_2$, 1 mM DTT, 40 μM Na_3VO_4, 0.1 μM poly-D-lysine and 0.005% Tween 20. Thereafter, the reaction was initiated by adding 150 nM of the substrate biotinyl-βAβAβAAAEEEYFFLFAKKK and 0.6 μM ATP, and the mixture was incubated for 30 min at room temperature. For control basal measurements, the enzyme was omitted from the reaction mixture. Following incubation, the reaction was stopped by adding 33 mM EDTA. The fluorescence acceptor (XL665-labelled streptavidine) and the fluorescence donor (anti-phospho-tyrosine-66K antibody labelled with europium cryptate) were then added. After 60 min, the fluorescence transfer was measured at

λ_{ex} = 337 nm, λ_{em} = 620 nm and λ_{em} = 665 nm by using a microplate reader (Rubystar, BMG). The enzyme activity was determined by dividing the signal measured at 665 nm by that measured at 620 nm. The results were expressed as a percent inhibition of the control enzyme activity.

The FGFR1 inhibitor staurosporine was used as a reference compound.

In-vitro antiangiogenic assay

In-vitro antiangiogenic properties of compound **11** were evaluated on human umbilical vein endothelial cells (HUVEC) cultured in an optimized medium containing the angiogenic factors bFGF and vascular endothelial growth factor (VEGF) (Lonza, Walkersville, MD, USA). Cells were resuspended in culture medium, seeded at 2×10^3/well in a 96-well plate precoated with 1% gelatin and treated with compound **11** at 0.01–50 μM for 24, 48 and 72 h. Following drug exposure, WST-1 was added and the absorbance measured.

Statistical analysis

Data are reported as the mean values ± s.e.m. of three measurements. Data analysis was performed with GraphPad Prism software, version 4.00. The 50% inhibitory concentration of cell growth (IC50) was calculated with a non-linear least squares curve using a sigmoidal dose–response (variable slope) equation. Statistical analyses were performed by using the Kruskal–Wallis test followed by the Dunn's test for multiple comparisons or the Mann–Whitney test (only for data reported in Table 2 and Figure 5). Significance was assumed at $P < 0.05$.

Results

Chemistry

The *(E)*-oxime ethers **1–16** (Table 1) were prepared starting from the previously described *(E)*-oximes (**17** and **18**)[9–12] following the procedure reported in Scheme 1. The reaction of *(E)*-oxime **17** at 120°C with an excess of the appropriate commercially available amine gave the amine derivates **19–25** (Figure 1). Subsequent reaction of **19–25** or **18**, as the sodium salt in anhydrous DMF with the appropriate arylmethylhalogenide afforded the desired *(E)*-oxime ethers **3–16** and **1** and **2**, respectively. The structure of all synthesized compounds was confirmed by examination of their ^{1}H NMR spectra. The *(E)* configuration of oximes **17–25** and oxime ethers **1–16** was assigned on the basis of the chemical shift (7.81–8.17 ppm) of the aromatic H_5 proton in accordance with what had been observed in previously described analogous compounds.[13]

Table 2 Comparison between in-vitro antitumour activities of compound **11** and 5-fluorouracil on MIA PaCa-2 cells

Time (h)	IC50 (μM)		E_{max} (% of control)[a]	
	11	5-fluorouracil	11	5-fluorouracil
24	22 ± 3.9*	>50	21 ± 3.4*	81 ± 0.6
48	18 ± 1.2	14 ± 1.1	2.6 ± 0.2*	31 ± 2.9
72	11 ± 0.5	5.3 ± 1.1	0.2 ± 0.2*	19 ± 3.0

[a]Maximum effect (E_{max}) was expressed as the lowest cell viability value after treatment, as compared to control (untreated samples). Changes in the mean IC50 or E_{max} values obtained at each exposure time that are significant (compound **11** versus 5-fluorouracil, $*P < 0.05$; Mann–Whitney test). Data are mean ± SEM of three measurements.

Biological evaluation

Cytotoxicity of the compounds on MIA PaCa-2 cells

Preliminary cytotoxicity evaluation showed that the antitumour activity *in vitro* was dependent on concentration and time of exposure to compounds (data not shown). The concentration that decreased cell viability by 50% (IC50) after 72 h was then calculated to better define the structure–activity relationship (Table 1). The increase in the mean IC50 values of compounds **3** to **6** suggested that the nature of the substituent on the phenyl group bound to the methylenoxyiminic linker moiety could be relevant for antitumour activity. Most importantly, compounds with an aminoalkyl chain at the 7-position were more active than compounds with a methyl group in the same position (see compounds **3–6** versus **1, 2**; Table 1). The number of carbon atoms in the aminoalkyl chain did not seem to be important for biological activity, since no substantial difference in the IC50 values of compounds **3**, **7** and **9** was observed. While the substitution of the aminoalkyl chain at the 7-position with a cyclic amine (piperidine or piperazine) led to a partial decrease in compound activity (see compounds **7** versus **14**), the introduction of a diaminoalkyl chain further ameliorated the anticancer profile of compounds, with a decrease in the mean IC50 value from 20 ± 1.6 to 12 ± 1.9 μM (compounds **3** and **10**, respectively) (Table 1).

Although compounds **10**, **11** and **12** displayed similar IC50 mean values after 72 h of exposure, compound **11** was the most active after 24 and 48 h (data not shown), therefore it was selected for further evaluation.

Compound 11 versus 5-fluorouracil

The progressive reduction in the mean IC50 values indicated that the antiproliferative effect of compound **11** increased from 24 to 72 h (Table 2), and the marked drop in cell proliferation caused by this compound was already evident 24 h after exposure (Figure 2). In this setting, compound **11** was significantly more active than 5-fluorouracil, both in terms of potency (IC50) and efficacy (E_{max}) (Table 2). Although after 48 and 72 h their potency was comparable, efficacy was significantly higher in cells treated with compound **11** than those given 5-fluorouracil (Table 2).

The effect of compound **11** and 5-fluorouracil were also evaluated against non-cancerous human oral epithelial cells at the maximum concentration tested on MIA PaCa-2 cells (i.e. 50 μM) for 72 h. 5-fluorouracil decreased cell viability by 59%, as compared to untreated cells ($P < 0.05$; Figure 3), while after treatment with compound **11** such a value did not differ significantly over control (–35%; Figure 3). It is noteworthy that compound **11** seemed to exhibit some selectivity for cancer cells since, in this experimental condition, almost 100% cell death was observed in the MIA PaCa-2 cell line.

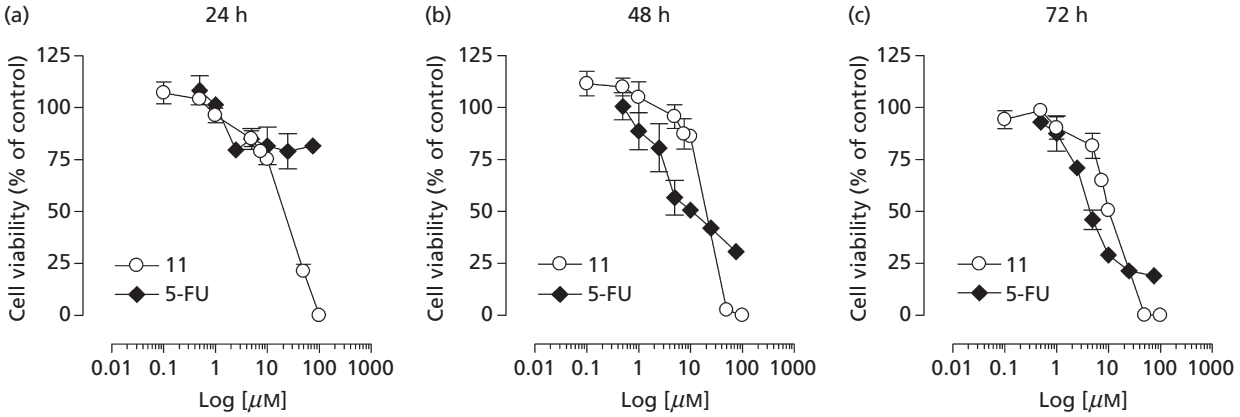


Figure 2 Response curves of MIA PaCa-2 cells to compound **11** and 5-fluorouracil. The response is shown after 24 (a), 48 (b) and 72 (c) h. Data are expressed as means ± SEM of three measurements. 5-FU, 5-fluorouracil.

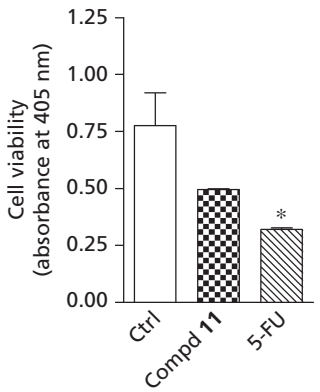


Figure 3 Effect of compound **11** and 5-fluorouracil on non-cancerous human oral epithelial cells. Effect is shown after exposure at 50 μM for 72 h. Data are expressed as means ± SEM of three measurements. 5-FU, 5-fluorouracil.

Apoptosis

To explore whether compound **11** was able to promote apoptosis, caspase 3/7 stimulation and quantification of histone-complexed DNA fragments were investigated in MIA PaCa-2 cells. After treatment with compound **11** at 20–100 μM, the extent of apoptosis (accumulation of oligonucleosomes in the cytoplasmic fraction) as well as caspase induction were proportional to the dose (Figure 4).

Growth stimulation and FGFR-1 kinase studies

Growth inhibition studies were also performed on MIA PaCa-2 cells grown in the presence of bFGF. Manipulating MIA PaCa-2 growth medium in this manner created circumstances in which cell growth was stimulated exclusively by bFGF. Under these conditions, compound **11** applied at 20 μM for 10 h specifically inhibited the growth of bFGF-stimulated MIA PaCa-2 cells by 72.8 ± 2.3% ($P < 0.05$) without affecting the growth of unstimulated cells (Figure 5). It is noteworthy that when tested at the same concentration on normal bronchial smooth muscle cells stimulated with bFGF, compound **11** also significantly decreased cell viability (−15% over control; $P < 0.05$) but the magnitude of the effect was much less pronounced than that observed on MIA PaCa-2 cells (Figure 6).

Investigation of the possible mechanism of action of compound **11** was performed by using the human FGFR-1 kinase assay on MIA PaCa-2 cells. The concentration–response curve in the range of 0.03–100 μM showed a modest decrease in FGFR-1 activity only at the highest tested concentration (80.2 ± 0.2% of control values; Figure 7).

In-vitro antiangiogenic effect

Compound **11** was tested on HUVEC stimulated with the angiogenic factors, bFGF and VEGF. The dose–response curve showed a time-dependent effect with the consequent decrease in mean IC50 values from 24.5 ± 1.1 μM to 15.8 ± 1.2 μM and 9.3 ± 1 μM from 24 to 48 h and 72 h, respectively ($P < 0.05$).

Discussion

Pancreatic cancer is a lethal malignancy that is highly resistant to the cytotoxic effects of radio- and chemotherapy. In our study, we carried out a cytotoxicity screening of newly synthesized naphthyridine derivatives on the poorly differentiated human pancreatic cancer cell line, MIA PaCa-2, a well-recognized chemoresistant in-vitro model which has already been used to test novel therapeutic agents.[2] The heterocyclic derivates **1–16** showed a good antitumour activity *in vitro*, particularly after 72 h of exposure. The structure–activity relationship suggests that the nature of the 7-substituent on the heterocyclic core as well as that of the substituent on the phenyl group bound to the methylenoxyiminic linker appear to be essential for anticancer activity.

The screening assay allowed us to select compound **11** as the most active one. The in-vitro antitumour effect of this compound was then compared with that of 5-fluorouracil, a drug commonly used in the management of pancreatic cancer patients.[14] Our results showed time-dependent cell killing by 5-fluorouracil, with the maximum effect observed after 72 h, a pharmacological profile that fits well with the molecular mechanism of this S-phase-specific chemotherapeutic agent. It is worth mentioning that after 24 h

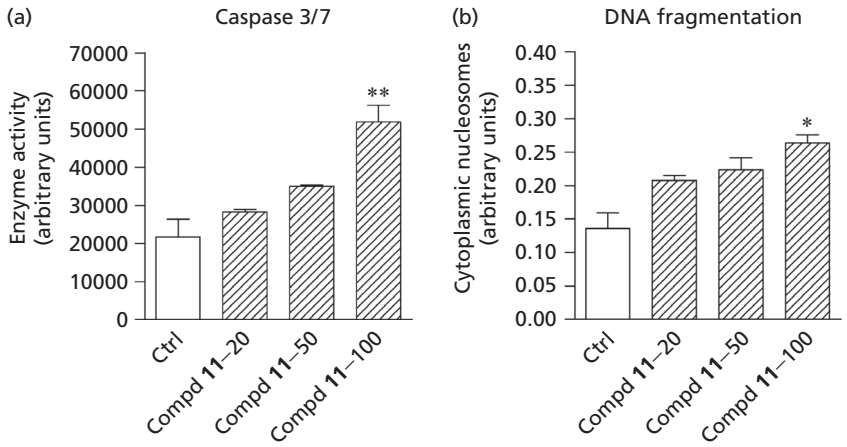


Figure 4 Induction of caspase 3/7 activity (a) and oligonucleosomal DNA fragmentation (b) after compound **11** at 20, 50 and 100 μM for 24 h. $^*P < 0.05$, $^{**}P < 0.01$ compared to the control sample (vehicle alone). Data are expressed as means ± SEM of three measurements.

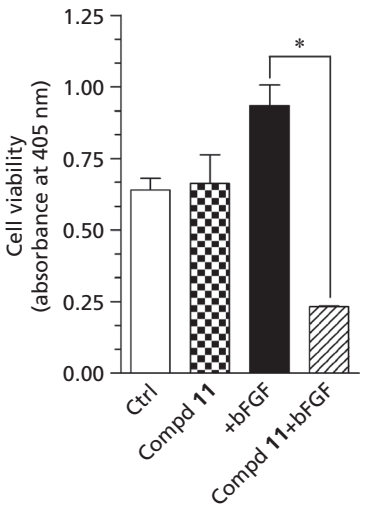


Figure 5 Effect of 20 μM compound **11** on basic fibroblast growth factor stimulation of MIA PaCa-2. $^*P < 0.05$, as compared to control values. bFGF, basic fibroblast growth factor.

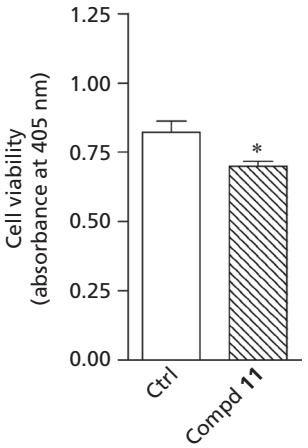


Figure 6 Effect of 20 μM compound **11** on human bronchial smooth muscle cell growth. $^*P < 0.05$, as compared to control values.

exposure, the majority of cells have not entered the cell cycle (there is a doubling time of MIA PaCa-2 cells of ~ 40 h); in this setting, 5-fluorouracil was substantially inactive while treatment with compound **11** resulted in 80% cell death. This notion, together with evidence demonstrating that doubling the exposure time to compound **11** increased efficacy tenfold, seemed to suggest that both cytotoxic and antiproliferative effects may account for its antitumour activity.

Compound **11** appeared to be less toxic than 5-fluorouracil against non-cancerous human oral epithelial cells and exhibited some selectivity towards cancer cells. However, this observation should be treated cautiously because *in vitro*, direct comparison of the therapeutic effect of novel compounds in normal versus cancer cells may result in misleading results with respect to the in-vivo setting. The therapeutic index may be a better predictor of in-vivo safety of an experimental compound and investigation of this possibility has been identified as an important area for future research into compound **11**.

Apoptosis is characterized by activation of caspase enzymes and cleavage of the genomic DNA into discrete fragments prior to membrane disintegration. Compound **11** displayed a similar dose-dependent ability to induce stimulation of caspase 3/7 activity and production of cytosolic histone-associated DNA fragments. It is thus conceivable that apoptotic cell death may be mediated via a caspase-dependent mechanism. These findings appear to be important when considering that the development of mechanisms that resist apoptosis confers high survivability and low drug sensitivity on pancreatic tumour cells.[2,15]

Several lines of evidence demonstrate that different cytokines, such as bFGF, and various cell-surface tyrosine kinase receptors, including the type I high-affinity FGFR-1, are overexpressed in pancreatic cancer and contribute to accelerated tumour growth.[16–18] Therefore, we carried out experiments that aimed to discover if biological response to compound **11** could be affected by the addition of exogenous

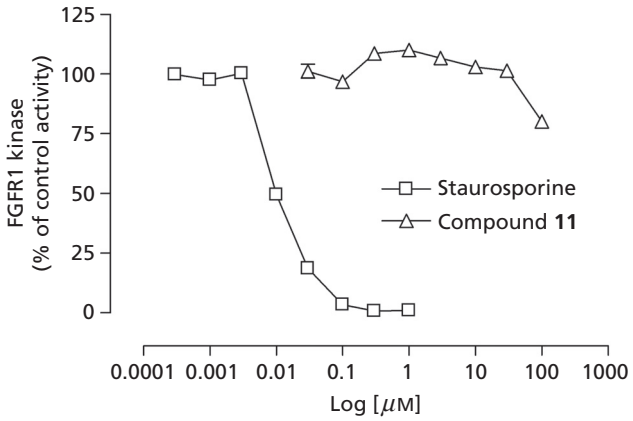


Figure 7 Changes in the activity of the human fibroblast growth factor receptor type 1 kinase by compound **11** and staurosporine. Data are expressed as means ± SEM of three or four measurements. FGFR, fibroblast growth factor receptor.

bFGF. Our findings confirm the mitogenic potential of bFGF in MIA PaCa-2 cells and demonstrate the ability of bFGF to sensitize tumour cells to the cytotoxic effect of compound **11**. However, receptor targeting appears to be partially involved in such an effect because significant inhibition of the human FGFR1 kinase *in vitro* was observed only at the highest concentration tested.

Although the precise mechanism underlying compound **11** cytotoxicity remains therefore to be clarified, the possible interference with downstream signalling molecules of the FGF/FGFR1 pathway should be taken into account. In line with this proposal, it has been recently reported that some naphthyridine derivatives act as allosteric inhibitors of the serine/threonine kinase Akt,[19] which is a pivotal cytoplasmic effector of FGF signalling and plays a role in cancer cell growth and survival.[20]

Tumour angiogenesis starts with cancerous tumour cells releasing molecules including VEGF and FGF which interact with transmembrane tyrosine kinase receptors displayed by endothelial cells.[21] The direct inhibition of vascular endothelial cell proliferation has been recognized as an appropriate model for the in-vitro investigation of antiangiogenic action.[22] We demonstrated that compound **11** markedly reduced human endothelial cell proliferation stimulated with bFGF and VEGF, in a concentration- and time-dependent manner. It is of note that the magnitude of the effect was comparable to that observed on MIA PaCa-2 cells, suggesting that the antiangiogenic activity may significantly contribute to the anticancer properties of the compound.

Conclusions

Naphthyridine derivates **1–16** promote cytotoxic effects against human pancreatic cancer cells in the micromolar range. Biological characterization of the most active compound provided the following pieces of evidence: (1) higher in-vitro efficacy and less toxicity than 5-fluorouracil, (2) induction of apoptosis, (3) enhancement of the antitumour effect in the presence of bFGF, (4) modest inhibition of FGFR1 kinase activity and (5) the ability to exert antiangiogenic effects. In conclusion, among the 16 substituted 2,3-dihydro-1,8-naphthyridines screened, compound **11** appears to be an interesting new prototype compound worthy of being explored as a promising antitumour agent. In this respect, further investigation of the mechanism of action of this compound is in progress.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

Funding

This research received no specific grant from any funding agency in the public, commercial or not-for-profit sectors.

Acknowledgement

We would especially like to thank Professor Gabriele Massimetti for helpful comments and assistance with statistical analysis.

References

1. Chari ST. Detecting early pancreatic cancer: problems and prospects. *Semin Oncol* 2007; 34: 284–294.
2. Yang L *et al*. Coexistence of high levels of apoptotic signaling and inhibitor of apoptosis proteins in human tumor cells: implication for cancer specific therapy. *Cancer Res* 2003; 63: 6815–6824.
3. Rocha-Lima CM. New directions in the management of advanced pancreatic cancer: a review. *Anticancer Drugs* 2008; 19: 435–446.
4. Litvinov VP. Chemistry and biological activities of 1,8-naphthyridines. *Russ Chem Rev* 2004; 73: 637–669.
5. Srivastava S *et al*. Synthesis and structure-activity relationships of potent antitumor active quinoline and naphthyridine derivatives. *Anticancer Agents Med Chem* 2007; 7: 685–709.
6. Tomita K *et al*. Synthesis and structure-activity relationships of novel 7-substituted 1,4-dihydro-4-oxo-1-(2-thiazolyl)-1,8-naphthyridine-3-carboxylic acids as antitumor agents. Part 1. *J Med Chem* 2002; 45: 5564–5575.
7. Deady L *et al*. Synthesis and cytotoxic activity of carboxamide derivatives of benzo[b][1,6]naphthyridin-(5H)ones. *Bioorg Med Chem* 2005; 13: 1341–1355.
8. Imperio D *et al*. Replacement of the lactone moiety on podophyllotoxin and steganacin analogues with a 1,5-disubstituted 1,2,3-triazole via ruthenium-catalyzed click chemistry. *Bioorg Med Chem* 2007; 15: 6748–6757.
9. Jamart-Gregoire B *et al*. A new series of tricyclic (aryloximino) propanolamines displaying very high selective beta 2-blocking properties. *J Med Chem* 1989; 32: 315–320.
10. Ferrarini P *et al*. Synthesis and β-blocking activity of (E)- and (Z)-iminoethers of 1,8-naphthyridine. Potential antihypertensive agents. *Eur J Med Chem* 1990; 25: 489–496.
11. Ferrarini P *et al*. Stereoselective synthesis and β-blocking activity of substituted (E)- and (Z)-4(1H)-[1-(3-alkylamino-2-hydroxypropyl)oximino]-2,3-dihydro-1,8-naphthyridine. Potential antihypertensive agents. *Eur J Med Chem* 1997; 32: 955–963.
12. Saccomanni G *et al*. Synthesis and beta-blocking activity of (R,S)-(E)-oximeethers of 2,3-dihydro-1,8-naphthyridine and

2,3-dihydrothiopyrano[2,3-b]pyridine: identification of beta 3-antagonists. *Bioorg Med Chem* 2003; 11: 4921–4931.
13. Ferrarini P *et al*. Synthesis and beta-blocking activity of (R,S)-(E)-oximeethers of 2,3-dihydro-1,8-naphthyridine and 2,3-dihydrothiopyrano[2,3-b]pyridine: potential antihypertensive agents – part IX. *Eur J Med Chem* 2000; 35: 815–826.
14. Neoptolemos J *et al*. A randomized trial of chemoradiotherapy and chemotherapy after resection of pancreatic cancer. *N Engl J Med* 2004; 350: 1200–1210.
15. Shi X *et al*. Acquired resistance of pancreatic cancer cells towards 5-fluorouracil and gemcitabine is associated with altered expression of apoptosis-regulating genes. *Oncology* 2002; 62: 354–362.
16. Yamanaka Y *et al*. Overexpression of acidic and basic fibroblast growth factors in human pancreatic cancer correlates with advanced tumor stage. *Cancer Res* 1993; 53: 5289–5296.
17. Douziech N *et al*. Growth effects of regulatory peptides and intracellular signaling routes in human pancreatic cancer cell lines. *Endocrine* 1998; 9: 171–183.
18. Kleeff J *et al*. Targeting of suicide gene delivery in pancreatic cancer cells via FGF receptors. *Cancer Gene Ther* 2002; 9: 522–532.
19. Bilodeau M *et al*. Allosteric inhibitors of Akt1 and Akt2: a naphthyridinone with efficacy in an A2780 tumor xenograft model. *Bioorg Med Chem Lett* 2008; 18: 3178–3182.
20. Hayashi H *et al*. Down-regulation of the PI3-kinase/Akt pathway by ERK MAP kinase in growth factor signaling. *Genes Cells* 2008; 13: 941–947.
21. Folkman J. Role of angiogenesis in tumor growth and metastasis. *Semin Oncol* 2002; 29: 15–18.
22. Zhong H. Bowen JP. Antiangiogenesis drug design: multiple pathways targeting tumor vasculature. *Curr Med Chem* 2006; 13: 849–862.