In-vitro antiproliferative activities and kinase inhibitory potencies of meridianin derivatives

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Marine alkaloid meridianin G derivatives, substituted on the pyrimidine ring by aryl groups, were evaluated for their kinase inhibitory potencies and their in-vitro antiproliferative activities. The derivatives were tested toward a panel of nine protein kinases (KDR, IGF-1R, c-Met, RET, c-Src, c-Abl, PKA, CDK2/cyclin A, and HER-1) and their in-vitro antiproliferative activities were evaluated toward a human fibroblast primary culture and two human solid cancer cell lines (MCF-7 and PA 1). Despite weak kinase inhibitory potencies, high in-vitro antiproliferative activities were found for compounds 5, 7, 12, and 14, which do not interfere with the PA 1 cell cycle and may be considered as direct cytolysis or apoptosis inducers. ***Anti-Cancer Drugs*** **19:789–792 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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Introduction

Cellular functions are extensively regulated by the wide family of protein kinases. The current state of human genome analysis reveals that there should be more than 500 different kinases. These enzymes exert various functions involved in signal transduction, cellular proliferation, cell motility, regulation of the cell cycle, and DNA damage response and repair. Because of their major role in cell development, protein kinases have become targets for cancer therapy. In fact, several kinase inhibitors are currently on the market, such as Gleevec (imatinib), Iressa (gefitinib), or the more recently FDA-approved Tykerb (lapatinib), Sutent (sunitinib), and Sprycel (dasatinib) [1].

Our investigations for the discovery of new kinase inhibitors led us to be interested in meridianins, a family of natural indolylpyrimidine derivatives isolated and characterized from the South Atlantic tunicate *Aplidium meridianum*. Seven meridianins (meridianins A–G) have been discovered so far (Fig. 1) [2–4]. Some of these meridianins or meridianin derivatives have been described as kinase inhibitors [4,5] or displayed antitumor activity [6]. Our purpose was to modify the molecular scaffold of meridianin alkaloids to improve their kinase inhibitory properties. Thus, we recently prepared meridianin G derivatives, substituted at the C-5′ position of the pyrimidine ring (Fig. 1) [7]. In a first approach, bromo derivative 1 was prepared and evaluated as a kinase inhibitor at a concentration of 10 µmol/l, and compared with meridianin G. We established that compound 1 was a considerably better inhibitor than its natural parent alkaloid, with a high inhibitory potency against diverse protein kinases. Consequently, we readily prepared other derivatives (compounds 2–15) substituted at the C-5′ position by various aryl groups. The indole nitrogen was substituted or not substituted by a methyl group.

In this article, the in-vitro antiproliferative activities and the kinase inhibitory potencies of compounds 1–15 are examined. The newly synthesized compounds were tested for their inhibitory potencies toward a panel of nine protein kinases: five receptor tyrosine kinases (KDR, IGF-1R, c-Met, RET and HER-1), two nonreceptor tyrosine kinases (Src and c-Abl), and two serine/threonine kinases (PKA and CDK2). Their antiproliferative activities were also examined *in vitro* by a fluorometric assay (resazurin reduction test) toward one human fibroblast primary culture and two human solid cancer cell lines: MCF-7 (breast adenocarcinoma) and PA 1 (ovarian carcinoma). Moreover, flow cytometry analyses were performed on PA 1 cells for the most active compounds to determine their effect on the cell cycle.

Materials and methods

In-vitro kinase inhibition assays

The in-vitro assays were performed in 96-well plates (30 µl) at ambient temperature for 15–45 min using recombinant glutathione *S*-transferase-fused kinase domains (3–100 ng,

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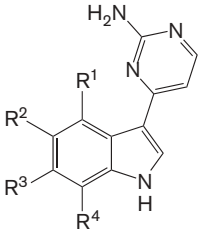
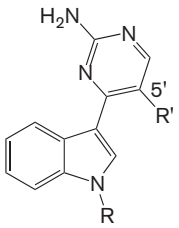
Fig. 1



meridianin A, R^1=OH, R^2=R^3=R^4=H
meridianin B, R^1=OH, R^2=R^4=H, R^3=Br
meridianin C, R^1=R^3=R^4=H, R^2=Br
meridianin D, R^1=R^2=R^4=H, R^3=Br
meridianin E, R^1=OH, R^2=R^3=H, R^4=Br
meridianin F, R^1=H, R^2=R^3=Br, R^4=H
meridianin G, R^1=R^2=R^3=R^4=H



1 R = H, R' = Br
2 R = H, R' = phenyl
3 R = CH_3, R' = phenyl
4 R = H, R' = 4-acetylphenyl
5 R = CH_3, R' = 4-acetylphenyl
6 R = H, R' = 4-biphenyl
7 R = CH_3, R' = 4-biphenyl
8 R = H, R' = 4-fluorophenyl
9 R = CH_3, R' = 4-fluorophenyl
10 R = H, R' = 2,4-difluorophenyl
11 R = CH_3, R' = 2,4-difluorophenyl
12 R = H, R' = 4-trifluoromethylphenyl
13 R = CH_3, R' = 4-trifluoromethylphenyl
14 R = H, R' = 4-trifluoromethoxyphenyl
15 R = CH_3, R' = 4-trifluoromethoxyphenyl

Meridianin A–G and meridianin G derivatives 1–15.

depending on specific activity) prepared previously [8–10]. [γ^{33}P]ATP was used as a phosphate donor and polyGluTyr-(4 : 1) peptide as an acceptor, with the exception of PKA, for which the heptapeptide Leu-Arg-Arg-Ala-Ser-Leu-Gly (known as Kemptide; Bachem, Bubendorf, Switzerland), was used as peptide substrate. Assays were optimized for each kinase using the following ATP concentrations: 1.0 µmol/l (c-Met and RET), 5.0 µmol/l (c-Abl), 8.0 µmol/l (KDR and IGF-1R), and 20.0 µmol/l (c-Src and PKA). The reaction was terminated by the addition of 20 µl of 125 mmol/l EDTA. Either 30 µl (c-Abl, c-Src, IGF-1R, RET) or 40 µl (all other kinases) of the reaction mixture was transferred onto immobilon–polyvinylidene difluoride membrane (Millipore, Bedford, Massachusetts, USA), pre-soaked with 0.5% H_3PO_4, and mounted on a vacuum manifold. Vacuum was then applied and each well rinsed with 200 µl of 0.5% H_3PO_4. The membranes were removed and washed four times with 1.0% H_3PO_4 and once with ethanol. Dried membranes were counted after mounting in a Packard TopCount 96-well frame and with the addition of 10 µl/well of Microscint. For RET kinase assay, either glutathione *S*-transferase-wild-type RET (15 ng) or glutathione *S*-transferase-RET-men2B protein (15 ng) was used. The CDK2/cyclin A assay was performed as described previously [11]. The IC_{50} values were calculated by linear regression analysis of the percentage inhibition.

In-vitro antiproliferative assays

Cell cultures

Stock cell cultures were maintained as monolayers in 75 cm^2 culture flasks in Glutamax Eagle's minimum essential medium with Earle's salts supplemented with 10% fetal calf serum, 5 ml of 100 mmol/l sodium pyruvate, 5 ml of 100X nonessential amino acids, and 2 mg gentamicin base. Cells were grown at 37°C in a humidified incubator in an atmosphere containing 5% CO_2.

Survival assays

Cells were plated at a density of 5×10^3 cells in 150 µl culture medium in each well of 96-well microplates and were allowed to adhere for 16 h before treatment with the tested drug. A stock solution 20 mmol/l of each tested drug was prepared in dimethylsulfoxide and kept at −20°C until use. Then 50 µl of each tested solution was added to the cultures. A 48-h continuous drug exposure protocol was used. The antiproliferative effect of the tested drug was assessed by the resazurin reduction test.

Resazurin reduction test

Plates were rinsed with 200 µl phosphate-buffered saline at 37°C and emptied by overturning on absorbent toweling. Then 150 µl of a 25 µg/ml solution of resazurin in minimum essential medium without phenol red was added to each well. Plates were incubated for 1 h at 37°C in a humidified atmosphere containing 5% CO_2. Fluorescence was then measured on an automated 96-well plate reader (Fluoroscan Ascent FL; Labsystems, Helsinki, Finland) using an excitation wavelength of 530 nm and an emission wavelength of 590 nm. In the conditions used, fluorescence was proportional to the number of living cells in the well. The IC_{50}, defined as the drug concentration required to inhibit cell proliferation by 50%, was calculated from the curve of concentration-dependent survival percentage, defined as fluorescence in experimental wells compared with fluorescence in control wells, after subtraction of the blank values.

Flow cytometric analyses

PA 1 cells were incubated with compounds 5, 7, 12, and 14 (at up to 5 µmol/l) or paclitaxel (1 µmol/l) for different periods of time (4, 24, and 48 h). The cells were harvested by trypsinization. The suspension containing both floating and adherent cells was spun once (400 *g*, 4 min, 4°C) in phosphate-buffered saline and the dry pellet was flash-frozen in liquid nitrogen. Then, the pellet was kept at −80°C until further analysis. The flow cytometric analysis of cell DNA content was performed using an Epics XL (Coulter, Hialeah, Florida, USA) after RNase treatment (1 mg/ml) and propidium iodide (50 µg/ml) labeling of cells. Fluorescence attributable to propidium iodide (wavelength 620 nm) was determined using excitation by an argon laser, operating at 488 nm and a power output of 15 mW. Cell distribution was calculated

using the Multicycle software program (Phoenix, Flow Systems, San Diego, California, USA).

Results and discussion

The kinase inhibitory potencies of compounds 1–15 were evaluated as IC_{50} values toward nine protein kinases (KDR, IGF-1R, c-Met, RET, c-Src, c-Abl, PKA, CDK2/cyclin A, and HER-1). None of the tested compounds was active toward c-Met. The more active compound tested was meridianin G bromo derivative 1, with IC_{50} values of 1.1 μmol/l toward KDR, 3.1 μmol/l toward IGF-1R, 7.8 μmol/l toward c-Abl, 2.5 μmol/l toward PKA, and 5.9 μmol/l toward CDK2/cyclin A (Table 1). Nevertheless, compound 1 was not active toward c-Met, RET, and HER-1. Besides c-Met, the other compounds were inactive toward KDR, c-Src, PKA, and CDK2/cyclin A. Compound 6 was inactive toward all the kinases tested whereas its N-methylated analog 7 was only weakly active toward RET ($IC_{50} = 7.8$ μmol/l). On the contrary, compounds methylated on the indole nitrogen and bearing a fluoro-substituted, trifluoromethyl-substituted, or trifluoromethoxy-substituted aryl group (9, 11, 13, and 15) were not active toward the kinases tested. Their counterparts, nonmethylated on the indole nitrogen (8, 10, 12, and 14), were weakly active toward HER-1 whereas compound 1 was not active against this kinase. Moreover, compounds 8 and 10 weakly inhibited c-Abl ($IC_{50} = 8.4$ μmol/l). The other compounds active toward HER-1 were compounds 2 and 3 with IC_{50} values of 5.0 and 9.1 μmol/l, respectively. These results indicate that methylation of the indole nitrogen is unfavorable to HER-1 inhibition but that, compared with compound 1, the presence of an aryl group at the 5′-position of the pyrimidine ring led to a gain of HER-1 inhibition. Compound 2 also showed mild inhibitory activities on RET ($IC_{50} = 8.9$ μmol/l) and c-Abl ($IC_{50} = 9.1$ μmol/l) kinases whereas its N-methylated analog 3 was also weakly active toward IGF-1R. Compound 4, substituted with a *p*-acetylphenyl group at the pyrimidine ring and its N-methylatyted analog 5 had a similar inhibition profile. They both inhibit IGF-1R and Ret with IC_{50} values in the range of 3–6 μmol/l. Moreover, compound 4 was weakly active toward c-Abl with an IC_{50} value of 7.1 μmol/l.

Surprisingly, referring to the weak kinase inhibitory potencies of compounds 2–15, some of them were highly cytotoxic. Indeed, high in-vitro antiproliferative activities were found for compounds 5, 7, 12, and 14, whereas meridianin G has been reported to be noncytotoxic [12]. High cytotoxicities were found toward PA 1 cell line for these compounds, with IC_{50} values in the range of 50–90 nmol/l, with the best in-vitro antiproliferative inhibitory potency for compound 5, with an IC_{50} value of 50 nmol/l. Weaker activities were found for compounds 4 ($IC_{50} = 2.0$ μmol/l) and 13 ($IC_{50} = 2.3$ μmol/l) toward PA 1. Compounds 5, 12, and 14 had good selectivity toward PA 1 cells compared with healthy fibroblast cells. On the contrary, compound 7 was also cytotoxic toward the fibroblast cell line with an IC_{50} value of 0.9 μmol/l. Compounds 5, 7, and 14 were also active toward MCF-7 cell line with IC_{50} values in the range of 0.28–1.6 μmol/l. The nature of the aryl group on the pyrimidine ring was crucial for high cytotoxicity. On the other hand, the best aryl groups are not of the same nature whether the indole nitrogen is substituted or not by a methyl group, indicating the importance of the substitution pattern on the C-5′ position of the pyrimidine ring and the indole nitrogen. In terms of cytotoxicity, among the non-N-methylated compounds, the best pyrimidine substituents appeared to be trifluoromethyl-substituted and trifluoromethoxy-substituted aryl groups (12 and 14, respectively). When indole nitrogen was methylated, the best cytotoxic agents were compounds 5 and 7, substituted on the pyrimidine ring by *p*-acetylphenyl and a biphenyl group, respectively. For these four compounds (5, 7, 12, and 14), the effect on the cell cycle was

Table 1 Inhibitory potencies toward a panel of protein kinases (IC_{50} in μmol/l) and antiproliferative activity of compounds 1–15 (percentage of residual activity of proliferation at 5 μmol/l–IC_{50} in μmol/l are indicated in brackets)

	Kinase inhibition IC_{50} in μmol/l									Antiproliferative activity % of residual activity at 5 μmol/l (IC_{50} in μmol/l)		
Compound	KDR	IGF-1R	c-Met	RET	c-Src	c-Abl	PKA	CDK2/A	HER-1	Fibro	MCF7	PA 1
1	1.1	3.1	>10	>10	ND	7.8	2.5	5.9	>10	76	78	33
2	>10	>10	>10	8.9	>10	9.1	>10	>10	5.0	72	47	52
3	>10	8.1	>10	>10	>10	>10	>10	>10	9.1	75	53	56
4	>10	5.9	>10	5.0	>10	7.1	>10	>10	>10	71 (9.5)	47 (22.7)	38 (2.0)
5	>10	3.4	>10	6.0	>10	>10	>10	>10	>10	25 (3.8)	30 (1.6)	4 (0.05)
6	>10	>10	>10	>10	>10	>10	>10	>10	>10	64 (8.0)	37 (16.4)	26 (15.8)
7	>10	>10	>10	7.8	>10	>10	>10	>10	>10	37 (0.9)	51 (0.28)	14 (0.08)
8	>10	>10	>10	>10	>10	8.4	>10	>10	6.1	87	77	69
9	>10	>10	>10	>10	>10	>10	>10	>10	>10	82	77	60
10	>10	>10	>10	>10	>10	8.4	>10	>10	7.4	90	75	83
11	>10	>10	>10	>10	>10	>10	>10	>10	>10	88	68	58
12	>10	>10	>10	>10	>10	>10	>10	>10	8.9	11 (8.7)	4 (14.0)	2 (0.09)
13	>10	>10	>10	>10	>10	>10	>10	>10	>10	15 (10.0)	10 (14.9)	2 (2.3)
14	>10	>10	>10	>10	>10	>10	>10	>10	5.2	13 (25.8)	5 (0.3)	0 (0.08)
15	>10	>10	>10	>10	>10	>10	>10	>10	>10	89	76	59

ND, not determined.

investigated by flow cytometry. No significant modification in the cellular percentage in each phase was observed according to control cells, except in the sub-G1 area.

Conclusion

High in-vitro antiproliferative activities were found for meridianin G derivatives 5, 7, 12, and 14, substituted by aryl groups at the C-5′ position of the pyrimidine ring, indicating that, depending on the substituents of the aryl group, an aromatic moiety at this position seems to be favorable for the biological activity. These cytotoxicities could not be directly correlated with the inhibitory potencies against the kinases tested. But, interestingly, compounds 5, 7, 12, and 14 exhibited high cytotoxicities toward PA 1. Moreover, compounds 5, 12, and 14 have shown good selectivity: they were particularly cytotoxic on PA 1 cells without significant growth inhibition on the healthy fibroblast cell line tested. The absence of a correlation between the kinase inhibition and cytotoxicity indicates that other cellular target(s) could be involved in the in-vitro antiproliferative activities of these meridianin derivatives. Additionally, compounds 5, 7, 12, and 14 do not interfere with PA 1 cell cycle and may be considered as direct cytolysis or apoptosis inducers. Further investigations are in progress to elucidate the cell-death mechanism induced.

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