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Meridianins, a new family of protein kinase inhibitors isolated from the Ascidian *Aplidium meridianum*

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Abstract—Meridianins are brominated 3-(2-aminopyrimidine)-indoles which are purified from *Aplidium meridianum*, an Ascidian from the South Atlantic (South Georgia Islands). We here show that meridianins inhibit various protein kinases such as cyclin-dependent kinases, glycogen synthase kinase-3, cyclic nucleotide-dependent kinases and casein kinase 1. Meridianins prevent cell proliferation and induce apoptosis, a demonstration of their ability to enter cells and to interfere with the activity of kinases important for cell division and cell death. These results suggest that meridianins constitute a promising scaffold from which more potent and selective protein kinase inhibitors could be designed.

1. Introduction

The phosphorylation of proteins on serine, threonine and tyrosine residues by the ~520 protein kinases encoded in the human genome constitutes one of the major mechanisms used by cells to regulate their metabolism and functions. The recent appreciation of the implication of abnormal protein phosphorylation in many human diseases has sparked considerable interest in the search for pharmacological inhibitors of kinases.[1] In addition to their potential applications in many therapeutic indications, these pharmacological inhibitors also constitute molecular tools, in more basic research, to probe the functions of specific kinases, to synchronize cells, to modify their differentiation fate, to alter their metabolism, etc. New chemical inhibitors are constantly being described and characterized with respect of their selectivity, mechanism of action, cellular effects, potential medical use.[2] In our laboratory we have focused our interest on a selection of kinases involved in cell cycle control and neuronal functions, namely the cyclin-dependent kinases (CDKs)[3,4] and glycogen synthase kinase-3 (GSK-3).[5] Numerous pharmacological inhibitors of CDKs have been discovered, characterized and recently reviewed.[6–8] In contrast, only very few GSK-3 inhibitors have been described.[9]

Marine organisms constitute a very promising and relatively poorly explored source of original bioactive molecules.[10] Among a huge diversity of structures, indole alkaloids are frequently found in marine invertebrates.[11,12] In this article we report on the identification of meridianins, a family of 3-(2-aminopyrimidine)-indoles, as potent inhibitors of various protein kinases. Meridianins were initially isolated from *Aplidium meridianum*, an Ascidian collected in the South Atlantic (South Georgia Islands).[13]

Keywords: Meridianin; Protein kinase; Cyclin-dependent kinase; Glycogen synthase kinase; Kinase inhibitor; Cancer; Neurodegenerative diseases.
Abbreviations: CDK, cyclin-dependent kinase; GSK-3, glycogen synthase kinase-3; MEM, Modified Eagle Medium; MTT, (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide; PBS, phosphate-buffered saline; SAR, structure/activity relationship.
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2. Meridianins, a family of kinase inhibitors

The meridianins have been first isolated from the marine Ascidian *Aplidium meridianum* (*Ascidiae*, *Polyclinidae* family),[14,15] collected at a depth of 100 m in the vicinity of the South Georgia Islands, South Atlantic.[13] These indoles substituted at C-3 with a 2-aminopyrimidine have now also been synthesized by two other groups.[16,17] While screening for new protein kinase inhibitors from natural sources, we discovered that meridianins (**1**–**7**) (Fig. 1) were potent inhibitors of several protein kinases. Kinase activities were assayed with appropriate substrates, with 15 μM ATP, and in the presence of increasing meridianin concentrations. IC_{50} values were estimated from the dose–response curves (Table 1). The compounds, except meridianin G and the related iso-meridianins C, were found to inhibit CDKs, GSK-3, PKA and other protein kinases in the low micromolar range. Meridianins B (**2**) and meridianin E (**5**) were the most potent inhibitors while meridianin G (**7**), isomeridianin C (**8**) and G (**9**)[18] were essentially inactive. Meridianins B and E were selected for further studies on selectivity and cellular effects.

We investigated the selectivity using meridianin E, the most CDK1-active meridianin, on 25 highly purified kinases (Table 2). Most kinases tested were inhibited in the 1–5 μM range, with the noticeable exceptions of Erk1, Erk2, MAPKK and Casein kinase 2 (IC_{50} values >100 μM). The most sensitive kinases were CDK1/cyclin B (0.18 μM), CDK5/p25 (0.15 μM), PKA (0.09 μM), PKG (0.60 μM), casein kinase 1 (0.40 μM) (Table 2).

3. Meridianins, structure–activity relationship

The very limited number of available meridianins precludes a detailed structure–activity relationship (SAR) study. However a few general features can be described. If we focus on CDK1 and CDK5, it is clear that a bromine substitution on position 7 of the indole (compare meridianins E and A or B) and a hydroxyl on position 4 (compare meridianins A and G, meridianins B and D) provide the best inhibitory activity. The removal of both bromine and hydroxyl substitutions (meridianin G) essentially inactivates meridianins as CDK inhibitors. A single bromine substitution in position 5 or 6 significantly increases the inhibitory activity (compare meridianins C or D and G). Two bromine substitutions somewhat reduce the inhibitory potency (compare meridianins C or D and F). Shifting the 2-aminopyrimidine from position 3 to position 2 inactivates the

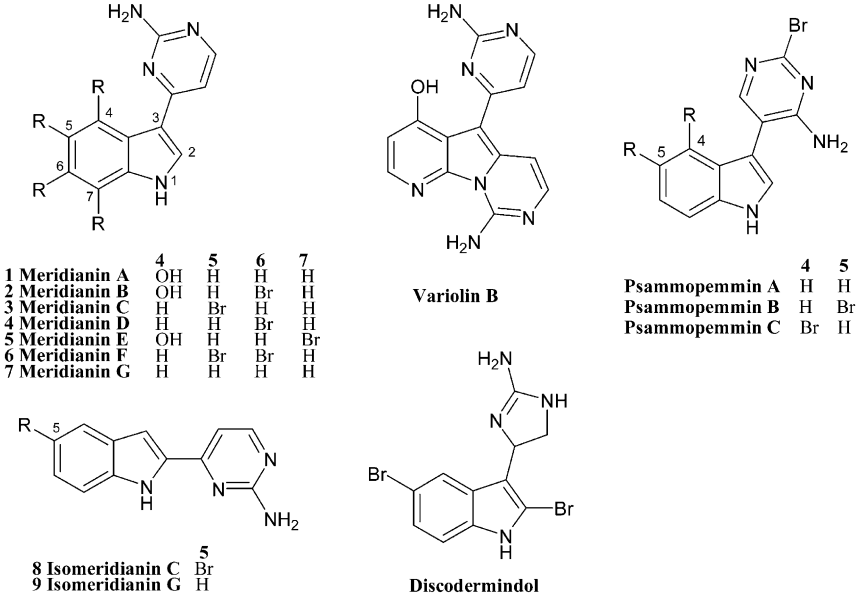

Figure 1. Structure of meridianins and related compounds.

Table 1. Effects of meridianins on the activity of a selection of protein kinases

Protein kinase	Roscovitine	Meridianin								
		A	B	C	D	E	F	G	Iso-C	Iso-G
CDK1/cyclin B	0.45	2.50	1.50	3.00	13.00	0.18	20.00	150.00	160.00	140.00
CDK5/p25	0.16	3.00	1.00	6.00	5.50	0.15	20.00	140.00	300.00	130.00
PKA	>1000	11.00	0.21	0.70	1.00	0.09	3.20	120.00	>1000	500.00
PKG	>1000	200.00	1.00	0.40	0.80	0.60	0.60	400.00	>1000	1000
GSK3-β	130.00	1.30	0.50	2.00	2.50	2.50	2.00	350.00	>1000	420.00
CK1	17	—	1.00	30.00	100.00	0.40	—	—	—	—

Enzyme activities were assayed as described in the Experimental, in the presence of increasing concentrations of meridianins. IC_{50} values, determined from the dose–response curves, are expressed in μM. —, not tested. The IC_{50} values for roscovitine [ref 21 and unpublished data] are indicated for comparison.

inhibitor (compare meridianin C and isomeridianin C). The hydroxyl group in position 4 seems to be important by itself for the inhibitory activity (compare meridianins A and G), but much less so when a bromine substitution is present (compare meridianins B and D).

If we focus on the cyclic nucleotide–dependent kinases, the structure/activity relationship is similar, but not identical: again a bromine substitution on position 7 of the indole and a hydroxyl on position 4, provide the best inhibitory activity. However the presence of two bromine (positions 5 and 6) does not significantly reduce the inhibitory activity.

Table 2. Inhibitory effects of meridianin E on the enzymatic activity of a panel of 25 protein kinases. IC_{50} values, determined from the dose–response curves, are expressed in μM

Protein kinase	IC_{50} (μM)
CDK1/cyclin B	0.18
CDK2/cyclin A	0.80
CDK2/cyclin E	1.80
CDK4/cyclin D1	3.00
CDK5/p25	0.15
Erk1	>100
Erk2	>100
c-Raf	1–10
MAPKK	>100
c-Jun N-terminal kinase	1.00
Casein kinase 1	0.40
Casein kinase 2	>100
Protein kinase C α	1.30
Protein kinase C β1	1.50
Protein kinase C β2	2.00
Protein kinase C γ	2.00
Protein kinase C δ	1.20
Protein kinase C ε	4.00
Protein kinase C η	1.30
Protein kinase C ζ	4.00
cAMP-dependent PK	0.09
cGMP-dependent PK	0.60
GSK3-α	0.90
GSK3-β	2.50
Insulin Receptor Tyr Kinase	80.00

It would be interesting to test meridianin-related compounds such as variolin B,[19] psammopemmins,[20] discodermindol[21] (Fig. 1) on protein kinases.

The available SAR, though very limited, suggests a possible binding mode of meridianins to the kinases. By analogy with the interaction of ATP-competitive inhibitors of CDKs,[7] it may be hypothesized that the primary amine of the pyrimidine and the hydroxyl of the indole act as H-bond donors while the nitrogen of the pyrimidine could act as a H-bond acceptor. Of course determination of the exact position and orientation of meridianins within the kinase will await co-crystallization. It may also vary according to the nature of meridianin and to the kinase.

4. Cellular effects of meridianins

The effects of meridianins A–F on cellular viability were tested on cell lines from human and murine origin. The compounds induced cytotoxic effects, which were time-, dose- and cell line-dependent. For example, the effect of a 40 h treatment on human teratocarcinoma NT2 cells survival is depicted in Figure 2. Clearly, only meridianins B and E (assayed at doses up to 10 μM) had an effect on NT2 proliferating cells, suggesting that only most kinase-active meridianins may display anti-proliferative properties. No effect was found when cells were treated with the vehicle (0.1% DMSO) alone (data not shown).

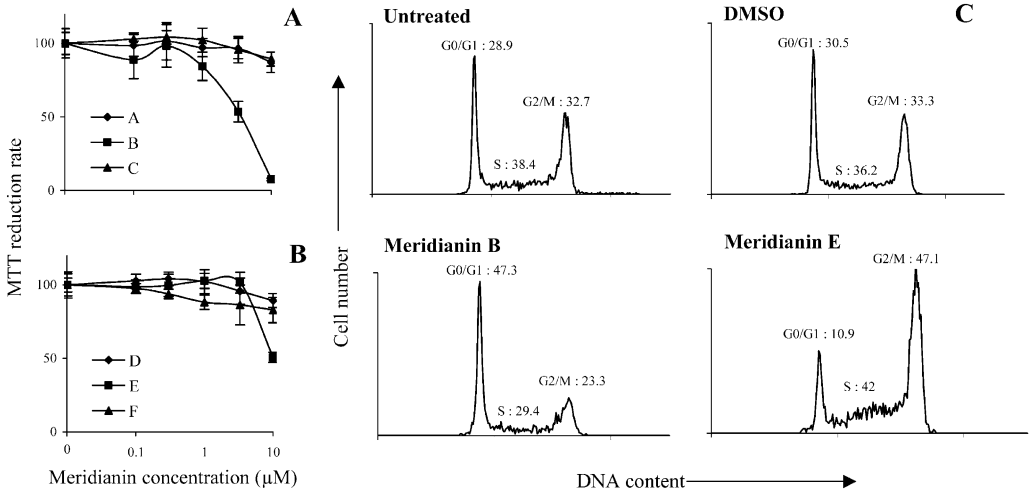

Figure 2. Anti-proliferative effects of meridianins. A & B, dose-dependent effects of meridianins A–C and D–F. NT2 cells were exposed to a range of concentrations of meridianins A–F for 40 h. Thereafter, cells were incubated with MTT and the anti-proliferative effect of meridianins was evaluated through the measurement of the inhibition of cellular reduction of MTT to MTT formazan, a reflection of the living cells density. Cell viability is expressed as percentage of the 0.1% DMSO-treated control. The values are given as the mean ± SD ($n = 8$). C, Cell cycle distribution of asynchronized NT2 cells treated for 15 h with meridianin B or E. Asynchronized cells (upper left) were treated for 15 h with the DMSO vehicle (upper right), with 10 μM meridianin B (lower left) and 10 μM meridianin E (lower right). The cell cycle distribution profile was analyzed by FACS.

In Table 3 we show the IC_{50} values of proliferation inhibition, as determined from the dose–response curves (10^{-3}–10^{2} μM) evaluated after a 72 h exposure to the compounds. It is important to note that the non-brominated alkaloid (meridianin A) was never cytotoxic, even when assayed at the highest dose, though it showed a relatively good inhibitory effect on various protein kinases. On the contrary, all the other meridianins (B–F) exhibited an evident cytotoxic activity at low μM range, though the cellular selectivity was strikingly different from one cell line to the other (Table 3). This may be reflecting variations in cellular permeability, in intracellular distribution, in metabolism of the meridianins and/or in diversity or availability of meridianins' molecular targets. Interestingly, the novel compounds were more cytotoxic to human or murine tumor cell lines than to the normal fibroblast cell line PTP (Table 3).

Regarding the relationship between meridianin cellular effects and their pharmacological activity, the capacity to inhibit the kinase activity of cAMP-dependent PK and cGMP-dependent PK seems to correlate best with their antiproliferative action, since only the non-cytotoxic meridianin A showed high IC_{50} values for these enzymes.

We next performed a flow cytometry analysis of unsynchronized NT2 cells following exposure for 15 h to 10 μM of meridianins B and E (Fig. 2C). The proliferation arrest induced by meridianin B in exponentially growing cells was clearly accompanied by an accumulation in G0/G1 phase whereas meridianin E treatment led to an arrest in the G2/M phase (Fig. 2C). In contrast, in the U937 cell line, meridianin B induced, after 28 h, an enhancement of cell distribution in S and G2/M phases and a massive accumulation in 'pre-G1' phase (54%), an indication of apoptosis (data not shown). These multiple arrest points, despite the relatedness of the active compound, are suggesting different intracellular targets. Apoptosis was further confirmed in the LMM3 cell line using the acridine orange staining. The apoptotic cells displayed a round morphology and very condensed nuclei with high intense fluorescence. Also, small membranous vesicles with condensed ADN content (apoptotic bodies) were seen (data not shown).

To test the reversibility of the cytotoxic effects induced by the meridianins, cells were treated with high doses of the compounds (IC_{100}). While the continuous presence of the compounds for a week led to apoptosis of all cells, removal of meridianins at 72 h and further culture in complete medium, allowed the regrowth of some cell colonies, suggesting a certain degree of cytostasis, even at high meridianin doses.

Table 3. Effects of meridianins A–F on cell proliferation

Cell Line	Meridianins					
	A	B	C	D	E	F
PTP	—	37.2	23.9	42.0	22.0	—
Hep2	NA	1.7	9.7	7.3	1.1	1.8
HT29	NA	—	5.5	36.6	—	—
RD	—	—	6.6	21.7	—	—
U937	—	11.6	2.7	16.9	9.8	0.2
LMM3	NA	17.7	9.3	33.9	11.1	1.4

Cell lines were cultivated as described in the Material and Methods section, in the presence of various concentrations of meridianins A–F, and the IC_{50} values (μM) of proliferation inhibition were determined from the dose–response curves. —, Not Tested; NA, No activity.

In conclusion we believe that meridianins, a family of marine natural products isolated from Ascidians, constitute a new scaffold of protein kinase inhibitors from which more potent and selective inhibitors can be designed.

5. Experimental

5.1. Natural products extraction and characterization

The green Ascidian *Aplidium meridianum* was collected by trawling at −100 m near the South Georgia Islands and stored at −20 °C until extraction. The frozen Ascidians were crushed and extracted three times with ethanol. The extract was dried under reduced pressure, and the yellow residue was flash-chromatographed on reverse phase Silica using a water/methanol gradient. Eluted fractions were chromatographed on Sephadex and further fractionated by HPLC.[13]

5.2. Kinase preparations and assays

CDK1/cyclin B, CDK2/cyclin A, CDK2/cyclin E, CDK4/cyclin D1 (provided by W. Harper), CDK5/p25 (vectors kindly provided by Dr. J. H. Wang), GSK-3β, His-tagged erk1 and erk2 (provided by M. Cobb), protein kinase C isoforms, the catalytic subunit of cAMP-dependent protein kinase (provided by S. Lohmann), cGMP-dependent protein kinase (provided by F. Hofmann or Sigma), casein kinases 1 & 2 (provided by L. Pinna), insulin receptor tyrosine kinase domain (CIRK-41) (provided by H. Y. L. Tung), c-raf, MAPKK (provided by D. Alessi), c-jun N terminal kinase (obtained from Promega) were assayed as described previously in the presence of a final ATP concentration of 15 μM.[22]

5.3. Cellular effects

5.3.1. Reagents. Penicillin, streptomycin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide (MTT), RNAse A, propidium iodide were purchased from Sigma.

5.3.2. Cell cultures. Clonal human NT2 teratocarcinoma cells were obtained from Stratagene (La Jolla, CA) and grown in Dulbecco's Modified Eagle Medium (MEM): Nutrient Mixture F-12 with 2 mM L-Glutamine (Bio Whittaker) supplemented with 5% FCS, penicillin (20 UI/mL) and streptomycin (20 μg/mL), at 37 °C, in a humidified atmosphere containing 5% CO_2 in air. Compounds were tested on several other cell lines: Hep-2 (larynx carcinoma), HT29 (colon carcinoma), RD (rhabdomyocarcinoma), U937 (myeloid leukemia) and PTP, a foreskin fibroblast cell line,

LMM3 (derived from the Balb/c transplantable mammary adenocarcinoma MM3).[23] These cells lines were cultured in MEM supplemented with 5% FCS, 2 mM L-glutamine, 80 μg/mL gentamycin. For the non-adherent U937 cells, RPMI medium plus 8% FCS, glutamine and gentamicin was employed. Quantification of cell number was made by hemacytometer counting. Viability was determined by the Trypan blue exclusion test.

5.3.3. Treatment with meridianins. Exponentially growing cells were incubated for 40 h with meridianins (stock solution dissolved in dimethylsulfoxide).

5.3.4. Cell viability assay. To quantify the effect of meridianins on cell viability, 0.2 mL of a cell suspension containing 3×10^4 viable cells/mL were seeded per triplicate in 96-well plates in complete medium. After 24 h cells were washed with PBS and incubation was continued in medium plus 2% FCS in the presence of different concentrations of the compounds (0.001–100 μM) or the vehicle DMSO (0.1–1%). Cell viability was evaluated at 40 or 72 h, by the MTS (Cell Titer 96TM, Promega), or MTT assay. The percentage of cell growth inhibition as (Absorbance in treated wells/Absorbance in control wells)×100 was calculated. The IC_{50} value was measured graphically from a dose–response curve constructed with at least 5 drug concentration points (all assays performed in triplicate and experiments repeated at least twice).

To study whether the compounds effects were reversible, log-phase growing cells were treated during 72 h with Meridianins B and C (IC_{100}) in the presence of 2% FCS. Then cells were carefully washed to remove the compounds and re-fed with fresh medium. As positive control, cells were continuously treated with the compounds. Cell growth was then monitored periodically using the MTS assay.

5.3.5. Cell cycle analysis by flow cytometry. NT2 cells were trypsinized, collected by centrifugation and fixed in cold 70% ethanol for at least 4 h. In the case of U937 cell line, treated or control cells were washed and collected by centrifugation. Fixed cells were washed in PBS, incubated with 10 μg RNAse A/mL and stained with 25 μg propidium iodide/mL for 1 h at 37 °C. The stained cells were then analyzed for cell cycle distribution on a FACSort flow cytometer (Becton Dickinson). Cell cycle analyses were performed as described using multiCYCLE (P Rabanovitch).[24]

5.3.6. Measurement of apoptosis. Apoptosis was evaluated by acridine orange (AO) staining. Subconfluent LMM3 cells were washed in PBS and treated with meridianins B or C (IC_{100}). At 48 h medium was removed and cells were stained with AO solution (10 μg/mL in PBS). Cell monolayers were immediately examined with a fluorescence microscope.

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