Piperlongumine: Tubulin-Destabilizing Agent?

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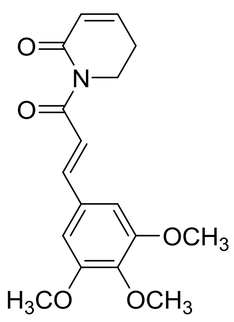
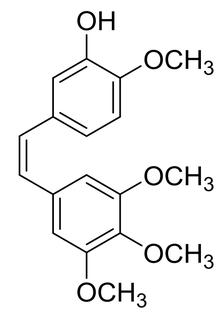
INTRODUCTION



Piperlongumine

Piperlongumine is a natural amide alkaloid isolated from the plant species Piper longum L. It is cytotoxic and has been reported to selectively kill cancer cells by targeting the stress response to **reactive oxygen species** (ROS). The use of small molecules to target cancer by altering cell levels of ROS is emerging area of research and there is huge potential for further exploration in this area.

Piperlongumine is structurally similar to a number of microtubule-destabilizing agents, including combretastatin A-4 and related methoxylated chalcones. This project focuses on the effects of piperlongumine on **tubulin** in breast cancer cells, a protein that is the main constituent of microtubules and is crucial for growth of tumours.



Combretastatin A-4

EFFECTS ON CELL CYCLE AND TUBULIN POLYMERISATION

Piperlongumine (PL) causes a decrease in the G_0/G_1 cell population and an increase in the G_2/M cell population of MCF-7 breast cancer cells at 24 and 48 hr at three concentrations (IC_{50} = 1.2 µM)(Figure 1). There was an increase in the percentage of apoptotic cells (sub-G_1) at concentrations of 10 and 20 µM piperlongumine after 48 hr. This profile is characteristic of microtubule-targeting agents.

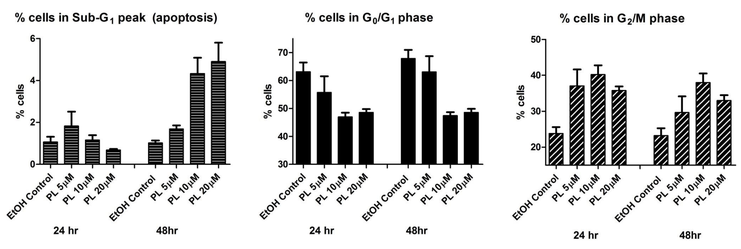
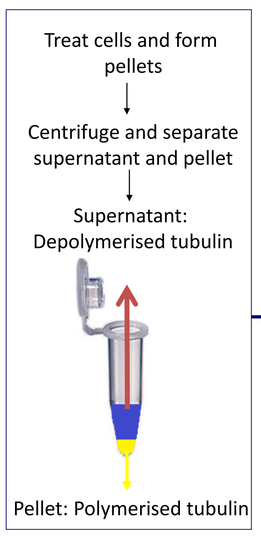


Figure 1. Effects of piperlongumine on the cell cycle and apoptosis in MCF-7 cells. Cells were treated with either vehicle [0.1% ethanol (v/v)] or piperlongumine (PL; 5, 10 or 20 µM) for 24 or 48 hr. Cells were fixed, stained with PI, and analyzed by flow cytometry.



Tubulin from piperlongumine-treated cells was detected almost wholly in the supernatant from MCF-7 cells, indicating microtubule deploymerization (Figure 2). These results indicate that piperlongumine is targeting the microtubules of MCF-7 breast cancer cells.

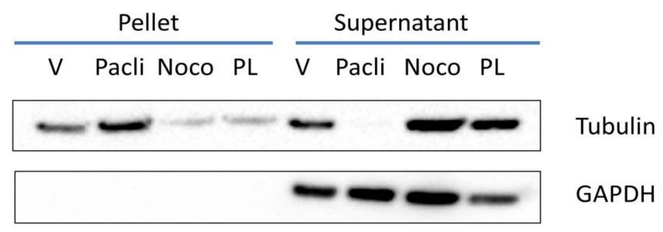
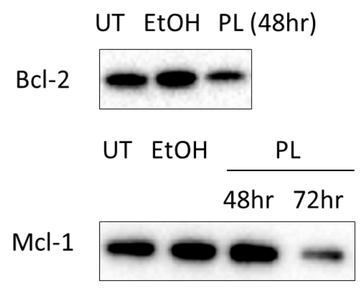


Figure 2. Effect of piperlongumine (PL) on the microtubule network of MCF-7 breast cancer cells was examined by a sedimentation assay and western blotting. Cells were treated with vehicle [V; 0.1% ethanol (v/v)], paclitaxel (Pacli; 1 µM), nocodazole (Noco; 1 µM) or PL (10 µM) for 4 hours. Unpolymerized and polymerized fractions were separated by centrifugation and collected as supernatant and pellet fractions.

EFFECT ON EXPRESSION OF ANTI-APOPTOTIC PROTEINS

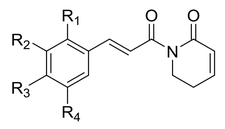


Anti-apoptotic proteins of the Bcl family contribute to an increased apoptotic threshold in cancer cells and allow cells to survive in stressful environments. The effects of piperlongumine (PL) on the expression of two anti-apoptotic proteins, **Bcl-2** and **Mcl-1**, were determined in MCF-7 cells.

Expression of **Bcl-2** decreased after 48 hr whilst that of **Mcl**-1 decreased at 72 hr. This indicates that PL induces apoptosis via the intrinsic pathway.

Figure 3. Piperlongumine (PL) downregulates the expression of anti-apoptotic proteins Bcl-2 and Mcl-1.
MCF-7 cells were treated with PL (10 µM). Untreated (UT) and vehicle (EtOH, 0.1 % v/v) controls were also examined. After the required time cells were harvested and separated by SDS-PAGE. The membrane was probed with anti-Bcl-2 or anti-Mcl-1 antibodies. Results are representative of three separate experiments.

PIPERLONGUMINE ANALOGUES



A series of mono-, di- and tri- and de-methoxylated analogues of piperlongumine were synthesised to study its structure-activity relationships.

Analogues **1**, **6** and **7** were the most potent in MCF-7 **breast cancer cells**, with similar IC_{50} values (table to the right). The main conclusions are:

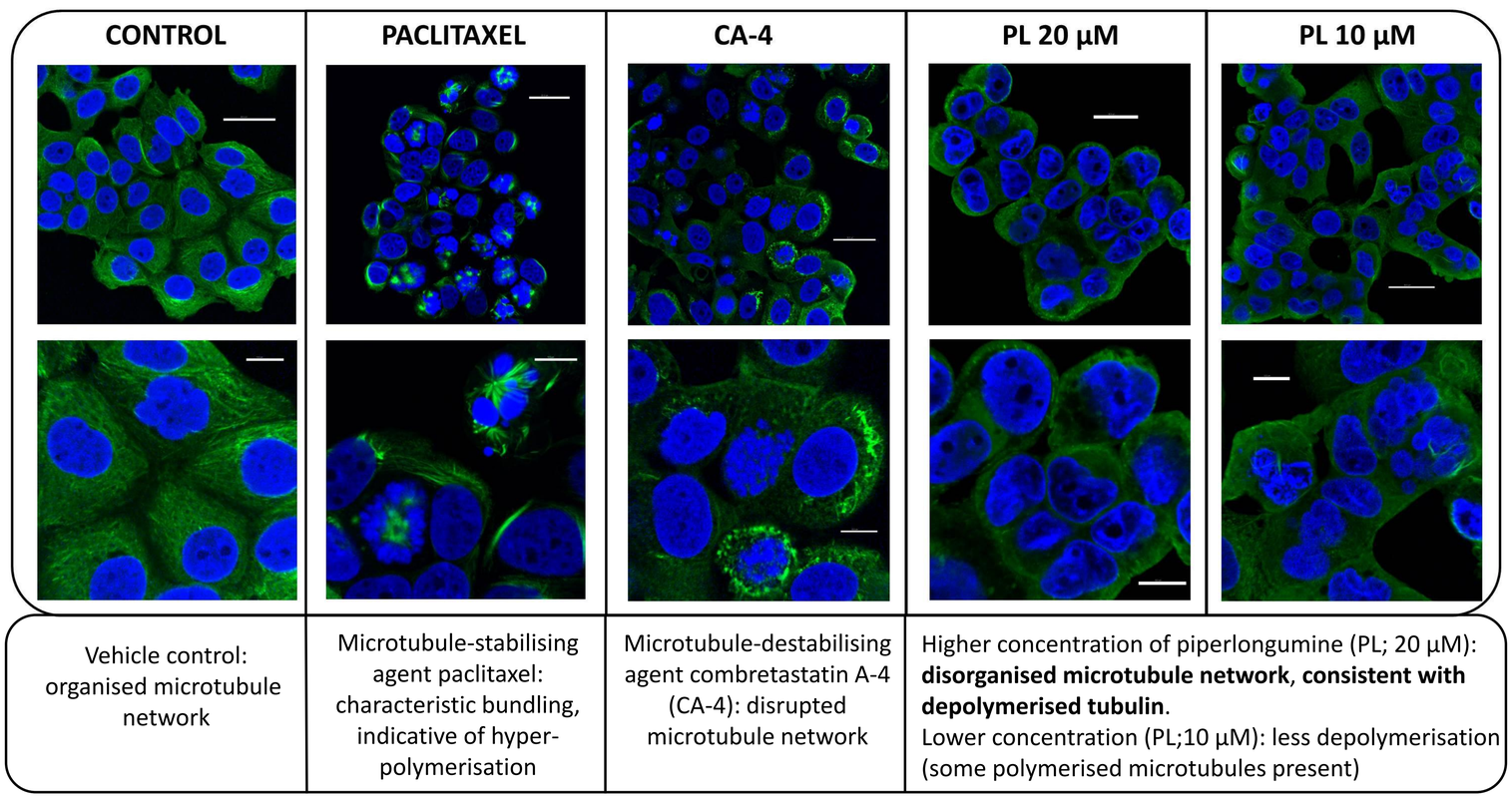
- Compounds with one methoxy group are less potent, regardless of the position of the group (**2**, **3**, **4**)
- Dimethoxy groups are best at positions 2 + 3 (**5** v's **6**)
- A 3,4,5-trimethoxy substitution, such as that found in naturally occurring piperlongumine, is most potent

Toxicity: All compounds (5 µM) except analogue **1** were relatively non-toxic to non-tumorigenic MCF-10a breast cells.

Analogue	R_1	R_2	R_3	R_4	IC_{50} (µM)[a]	% cell death[b]
1	H	H	H	H	3.5	20
2	OCH_3	H	H	H	9.6	0
3	H	OCH_3	H	H	7.5	10
4	H	H	OCH_3	H	8.2	0
5	OCH_3	H	OCH_3	H	8.5	0
6	H	OCH_3	OCH_3	H	3.7	12
7	OCH_3	H	OCH_3	OCH_3	3.4	12
Piperlongumine	H	OCH_3	OCH_3	OCH_3	1.2	nt

[a]IC_{50} values in MCF-7 cells at 48 hr
[b]cell death in MCF10a cells at 48 hr at 5 µM concentration
Both determined by alamarBlue assay; n=3

IMMUNOFLUORESCENCE AND CONFOCAL MICROSCOPY



Immunofluorescence in combination with confocal microscopy can be used to directly visualise the microtubule network in cells. The result provides additional evidence that piperlongumine targets tubulin, causing depolymerisation of microtubules (Figure 3).

Figure 3. Piperlongumine (20 µM) depolymerises the microtubule network of MCF-7 cells. MCF-7 cells were treated with vehicle control [1% ethanol (v/v)], paclitaxel (1 µM), CA-4 (100 nM) or piperlongumine (PL; 20 or 10 µM) for 16 h. Cells were fixed in 4% paraformaldehyde and stained with mouse monoclonal anti-α-tubulin–FITC antibody (clone DM1A) (green), Alexa Fluor 488 dye and counterstained with DAPI (blue). Images were captured by Leica SP8 confocal microscopy with Leica application suite X software. Representative confocal images of three separate experiments are shown. Scale bar: 30 µM (top images); 10 µM (bottom images).

CONCLUSIONS

- Piperlongumine induces G_2/M arrest and apoptosis, causes depolymerisation of α-tubulin in a sedimentation/Western blot assay, and disrupts the microtubule organisation in MCF-7 breast cancer cells as visualised by confocal microscopy.
- These results indicate the piperlongumine is a tubulin-destabilizing agent, in addition to its known effects as a reactive oxygen species (ROS) inducer.
- A series of structural analogues of piperlongumine were synthesised and assessed for their anti-proliferative effects. Their tubulin-depolymerising ability and induction of reactive oxygen species will be investigated to identify the best dual-acting compound as a candidate for further development.

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