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Novel inhibitors targeting PPM1D phosphatase potently suppress cancer cell proliferation

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ABSTRACT

Protein phosphatase magnesium-dependent 1δ (PPM1D, Wip1) is a p53 inducible serine/threonine phosphatase. PPM1D is a promising target protein in cancer therapy since overexpression, missense mutations, truncating mutations, and gene amplification of PPM1D are reported in many tumors, including breast cancer and neuroblastoma. Herein, we report that a specific inhibitor, SL-176 that can be readily synthesized in 10 steps, significantly inhibits proliferation of a breast cancer cell line overexpressing PPM1D and induces G2/M arrest and apoptosis. SL-176 decreases PPM1D enzyme activity potently and specifically in vitro. These results demonstrate that SL-176 could be a useful lead compound in the development of effective anti-cancer agents.

1. Introduction

The gene *PPM1D* encoding serine/threonine protein phosphatase PPM1D (also known as Wip1 and PP2Cδ) is located on the chromosomal region 17q23, which is frequently amplified in many cancers.[1] Overexpression of PPM1D and amplification of the *PPM1D* gene have been reported in various cancers, including breast cancer, hepatocellular carcinoma, pancreatic adenocarcinoma, ovarian carcinoma and neuroblastoma.[2–6] PPM1D was identified as a protein induced by p53 following activation in response to irradiation.[7] It has been reported that PPM1D regulates the p53 pathway in a negative feedback loop by dephosphorylating p53[8,9] and other important factors involved in cell cycle regulation.[9,10] Consequently, overexpression, missense mutations, and truncating mutations of PPM1D are associated with reduced activation of the regulatory factors and have been implicated in carcinogenesis.[11,12] Additionally, amplification of *PPM1D* and overexpression of PPM1D in these cancers are significantly associated with poor clinical prognosis. This suggests that analyzing alterations in copy number and expression level is important for proper clinical prognosis and selection of therapeutic options.[2,5,6] Various PPM1D inhibitors, including peptidic inhibitors derived from substrate sequences and small chemical inhibitors, were reported.[13–17] Among them, GSK2830371 was demonstrated very strong inhibition of cell growth in cell lines overexpressing PPM1D with wild-type *TP53*, including breast cancer and neuroblastoma.[13–17] However, cell lines with mutant *TP53* were shown to be resistant to GSK2830371. Because PPM1D overexpression has been observed in cell lines with *TP53* mutation, it is required to develop inhibitors that suppress cancer cell growth regardless of *TP53* status.[18] Recently, we demonstrated that the PPM1D inhibitor, SPI-001, suppressed the proliferation of a breast cancer cell line.[19] The combined administration of SPI-001 and doxorubicin suppressed the cell viability of colorectal carcinoma cells overexpressing C-terminal truncated PPM1D.[20] Thus, PPM1D is a therapeutic target and SPI-001 is a promising lead candidate for additional anti-cancer agents. However, SPI-001 is a challenging molecule to synthesize that involves 33 steps. Hence, analogs of SPI-001 that require simpler synthetic routes are more attractive and viable lead candidates. Here, we report that a novel PPM1D inhibitor, SL-176, effectively prevents cancer cell proliferation.

2. Results and discussion

We designed simplified and more compact analogs of SPI-001. These analogs, SL-175 and SL-176 were synthesized in 10 steps from a commercially available starting material (Scheme S1). Both analogs share a *trans* decalin framework and bulky hydrophobic

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groups. These analogs differ from the hydrophilic groups they bear wherein SL-175 features a hydroxyl functionality, in contrast to the carboxylic acid moiety of SL-176 (Fig. 1).

We examined the effect of these compounds on the proliferation of PPM1D overexpressing MCF-7 breast cancer cells (Fig. 2 and Table 1). The half maximal inhibitory concentration (IC_{50}) value of SL-176, which has a carboxyl group, for the growth inhibition was 7.4 μM. Also, IC_{50} values of SL-175, which has a hydroxyl group, SPI-001, and GSK2830371 for the growth inhibition were 36.1 μM, 26.9 μM, and 9.5 μM, respectively. SL-176 has three-times more potent inhibitory activity than SPI-001. SL-176 completely suppressed cell proliferation over 20 μM concentration. In contrast, GSK2830371 did not inhibit cell growth completely even in high concentration. SL-176 showed the strongest inhibition of MCF-7 proliferation. Next, the inhibitory activity of these compounds against PPM1D phosphatase activity was examined using recombinant His-PPM1D(1-420) and a phosphorylated peptide derived from p53 as the substrate (Fig. 3A and Table 1). The IC_{50} value of SL-176 against PPM1D phosphatase activity was 110 nM whereas GSK2830371 showed an IC_{50} value of 86.3 nM. SL-175 inhibited PPM1D dephosphorylation twice the IC_{50} of SL-176. These results indicated that SL-176 had extremely strong inhibitory effects on both in vitro and in vivo. In order to analyze the inhibitory activity of SL-176 in more detail, we examined the mode of enzyme inhibition (Fig. 3B). The data of SL-176 were fitted by global fitting analysis in noncompetitive inhibition. Moreover, to confirm specificity of SL-176 for PPM1D, we performed an in vitro phosphatase assay using three recombinant enzymes and phosphorylated peptides of their respective substrate (Fig. 3C). SL-176 inhibited His-PPM1D(1-420) phosphatase activity at submicromolar concentrations, whereas neither His-PPM1A, a PPM1 phosphatase family member, nor the non-PPM1 phosphatase family members, PP1 or PP2A, were inhibited by SL-176. This result showed that SL-176 had high specificity for PPM1D phosphatase in vitro. Next, we analyzed the PPM1D specificity at the cellular level using H1299, p53-null cell line, which expresses normal level of PPM1D, and PPM1D-overexpressed cell line, namely H1299 (PMD-9), which we established by transgenic HA-PPM1D (Fig. 3D and Fig. S1A). SL-176 showed a much more potent inhibition of cell proliferation against H1299(PMD-9) cells than the H1299 cells. On the other hand, GSK2830371 was not effective in growth inhibition of both H1299 and H1299(PMD-9) (Fig. S1B). These results suggested that SL-176 selectively inhibited cell proliferation in p53-null cells with overexpression of PPM1D.

The single administration of SL-176 induced an increase in the phosphorylation level of p53 at Ser15 in MCF-7 cells (Fig. 4A). In the absence of SL-176, the phosphorylation level of p53 slightly increased in response to UV-induced DNA damage in MCF-7 cells. As expected, the treatment of SL-176 with UV irradiation significantly increased the phosphorylation level of p53 and protein expression level of p53. This showed that SL-176 inhibited PPM1D in the cell and induced the activation of p53. It has been reported that up-regulation of phosphorylated p53 induces G2/M arrest and apoptosis.[21] Therefore, we analyzed the cell cycle of MCF-7 cells using flow cytometry (Fig. 4B). Treatment with SL-176 increased the amount of cells in the G2/M and sub-G1 phase. In SL-176-treated cells, the ratio of Annexin V positive cells was higher than control cells (Fig. 4C). These results suggested that SL-176 induced cell cycle arrest at G2/M and apoptosis via activation of p53 by PPM1D inhibition.

In cancer cells overexpressing PPM1D, the inactivation of p53 via hypophosphorylation was observed. The administration of a PPM1D inhibitor induces an increase in the phosphorylation level of p53 and the subsequent activation of p53 leads to growth inhibition. However, inhibition of cell proliferation was observed in a p53-null cell line where PPM1D was overexpressed in this report.

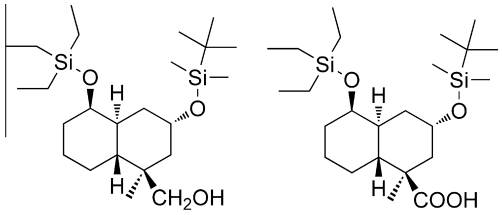

Figure 1. Chemical structures of SL-175 and SL-176.

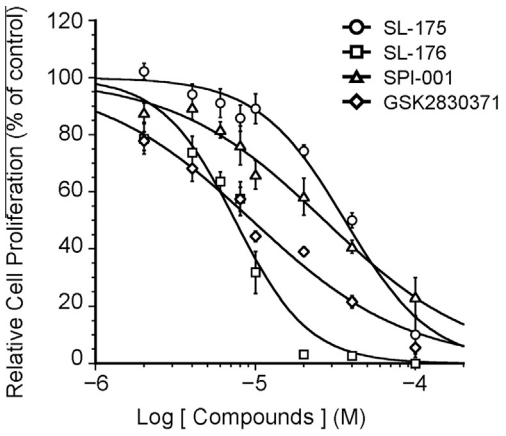

Figure 2. Growth inhibition of the compounds on PPM1D overexpressing cell line MCF-7. MCF-7 cells were incubated with each compound (circle, SL-175; square, SL-176; triangle, SPI-001; diamond, GSK2830371). Cell numbers were measured after 3 days. Data represent the mean ± SE, $n \geqslant 3$.

Table 1
The effects of the inhibitors on growth inhibition of MCF-7 cells and in vitro phosphatase activity of PPM1D

Compounds	Inhibitory activity	
	MCF-7 cell proliferation IC_{50}* (μM)	In vitro phosphatase activity IC_{50}* (nM)
SL-175	36.1 ± 2.89	215 ± 28.8
SL-176	7.4 ± 0.72	110 ± 12.9
SPI-001	26.9 ± 2.07	86.9 ± 8.43
GSK2830371	9.5 ± 0.86	86.3 ± 8.80

* The IC_{50} value of each compound against in vitro phosphatase assay using His-PPM1D(1-420) and MCF-7 cell proliferation. The standard errors of fittings are indicated.

Specifically, Natrajan et al. reported that a PPM1D inhibitor represses the proliferation of p53-mutated cells in which PPM1D is overexpressed.[18] It has been suggested that a p53-independent pathway exists, which might explain why therapeutic agents targeting PPM1D can inhibit cell proliferation in p53-null cell, but this needs to be elucidated further.[22]

In this report, we clearly demonstrated that SL-176 is a highly potent inhibitor that can effectively suppress proliferation of PPM1D-overexpressing cancer cells, regardless of the p53 status. SL-176 completely suppressed over 20 μM concentration. GSK2830371 showed some inhibition in a low concentration; however, it did not inhibit cell growth completely even in high concentration. Therefore, the inhibition curve of GSK2830371 showed a mild shift (Fig. 2). The administration of GSK2830371 was reported to induce abolishment of PPM1D protein.[17,23] On the other hand, SL-176 did not affect the PPM1D level, probably resulting in the effective inhibition of cell proliferation.