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NOTE

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Decreased sensitivity of cyclin-dependent kinase 4/6 inhibitors, palbociclib and abemaciclib to canine lymphoma cells with high p16 protein expression and low retinoblastoma protein phosphorylation

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ABSTRACT. Canine lymphoma/leukemia cell lines with p16 protein expressions: high (17–71 and GL-1) and low (CLBL-1, CLC, Nody-1, and UL-1) were treated *in vitro* with cyclin-dependent kinase 4 and 6 (CDK4/6) inhibitors, palbociclib or abemaciclib. Cell proliferation decreased as a result, with higher IC_{50} levels observed in the high p16 (17–71 and GL-1) and one low p16 (UL-1) cell lines compared with the low p16 cells (CLBL-1, CLC, and Nody-1). As expected, palbociclib and abemaciclib treatment reduced pRb phosphorylation in a dose-dependent manner, especially in cells with low p16. These results suggest that CDK4/6 inhibitors have potential as new chemotherapeutic agents for canine lymphoma and high p16 protein expression may be used as a biomarker for resistance to CDK4/6 inhibitor therapy.

KEYWORDS: canine lymphoma, phosphorylated pRb (pRb-P), p16 (CDKN2A)

The endogenous cyclin-dependent kinase 4 and 6 (CDK4/6) inhibitor, p16 (also called CDKN2A), inhibits cyclin-CDK4/6 [4], resulting in G1-S cell cycle arrest by inhibiting the phosphorylation of retinoblastoma protein (pRb) [11]. Dysregulation of the p16-pRb pathway results in increased cell proliferation [12] and has been implicated as the impetus of many cancers [2, 5, 6, 17].

CDK4/6 inhibitors (CDK4/6i), namely palbociclib and abemaciclib, are novel effective therapies approved by the Food and Drug Administration (FDA) and European Medicines Agency (EMA) for the treatment of human breast cancers in combination with other therapeutics [21, 22]. Their inhibitive properties affect cancer cell proliferation, blocks the progression from G1 to S phase, and may inhibit the metastatic potential of cancer cells [14]. In human cancer cell lines, it has been reported that CDK4/6i can effectively inhibit the proliferation of tumor cells that lose endogenous inhibition of CDK4/6 due to p16 methylation or deletion [15, 16]. In canine studies, CDK4/6i and palbociclib have antitumor effects on canine mammary tumor cells [25] and could potentially be used as new anti-cancer treatments for canine melanoma [3]; however, there is no data available simultaneously analyzing the expression of p16 protein and phosphorylated pRb (pRb-P).

Decreased expression of p16 has been shown to be common in established canine lymphoma cell lines and lymphoma cells obtained from naturally occurring clinical cases [7–10]. Recently, we have found that certain canine lymphoma cell lines simultaneously exhibit p16 gene methylation, loss of p16 protein expression, and pRb hyperphosphorylation [18], suggesting that the p16-pRb pathway is one of the most crucial mechanisms in canine lymphomagenesis. Moreover, we also found that the expression level of the p16 protein was more strongly correlated with the pRb-phosphorylation level than with p16 mRNA. Therefore, the current study focused on CDK4/6i

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(palbociclib and abemaciclib) for the treatment of canine lymphoma cells as potential new drugs and explored the correlation between drug sensitivity and expression levels of the p16 protein and pRb-P in canine lymphoma cell lines.

Six canine lymphoma/leukemia cell lines were used based on our previous study [18]: two cell lines showed high p16 protein expression (17–71 [23] and GL-1 [20]) and four cell lines showed low p16 protein expression (CLBL-1 [24], CLC [27], Nody-1 [26], and UL-1 [29]). All canine lymphoma cell lines were maintained in complete medium (RPMI-1640; FUJIFILM Wako Pure Chemical Corp., Tokyo, Japan) containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Nacalai Tesque, Kyoto, Japan) maintained at 37°C in humidified air containing 5% CO_2.

To explore the relationship between p16 protein expression and sensitivity to palbociclib and abemaciclib, we first examined the half-maximal inhibitory concentration (IC_{50}) of each drug using the cell counting kit-8 (CCK-8) assay (Dojindo Laboratories, Kumamoto, Japan). Each cell line was treated with palbociclib (Sigma-Aldrich, St. Louis, MO, USA) or abemaciclib (LKT Laboratories Inc., St. Paul, MN, USA) at various concentrations (0, 0.01, 0.1, 1, 10, and 100 µM) in 96-well plates (3 wells/group) for 48 hr. Briefly, cell proliferation was assessed by adding 10 µL WST-8 (2-[2-methoxy-4-nitrophenyl]-3-[4-nitrophenyl]-5-[2,4-disulfophenyl]-2H-tetrazolium, monosodium salt) and incubated for 4 hr. Absorbance was measured at a wavelength of 450 nm. The IC_{50} of each drug was calculated from drug survival curves (Fig. 1A–C). The IC_{50} values of palbociclib and abemaciclib were analyzed using one-way ANOVA and Tukey's *post-hoc* test. Statistical significance was set at $P<0.05$.

The proliferation of palbociclib- and abemaciclib-treated cells was lower in the cell lines with low p16 (CLBL-1, CLC, and Nody-1) than in those with high p16 (17–71 and GL-1) and one low p16 (UL-1) [Fig. 1A]. In cell lines with high p16 (17–71 and GL-1), the IC_{50} values of abemaciclib were 1.6378 ± 0.1374 µM and 0.4060 ± 0.0957 µM, respectively, and those of abemaciclib were 0.1316 ± 0.0142 µM and 0.0330 ± 0.0082 µM, respectively (Fig. 1B and 1C). In cell lines with low p16 (CLBL-1, CLC, Nody-1, and UL-1), the IC_{50} values of palbociclib were 0.0081 ± 0.0013, 0.0061 ± 0.0004, 0.0086 ± 0.0013, and 1.1742 ± 0.0844 (µM), respectively, and those of abemaciclib were 0.0053 ± 0.0005, 0.0068 ± 0.0003, 0.0089 ± 0.009, and 0.1128 ± 0.0018 (µM), respectively. In summary, the IC_{50} values of palbociclib and abemaciclib in cells with low p16 (CLBL-1, CLC, and Nody-1), except UL-1, were consistently lower than those in cells with high p16 (17–71 and GL-1), suggesting that cells with high p16 were relatively resistant to CDK4/6i. This is concordant with a previous human study reporting that IC_{50} values of the p16 unmethylated (p16 expressing) cell lines were consistently higher than those of the p16 methylated (p16 low-expressing) cell lines and p16 deleted cell lines (18.88 vs. 4.61 and 3.99 [µM]), respectively] in human lung and gastric cancer cell lines treated with palbociclib [16].

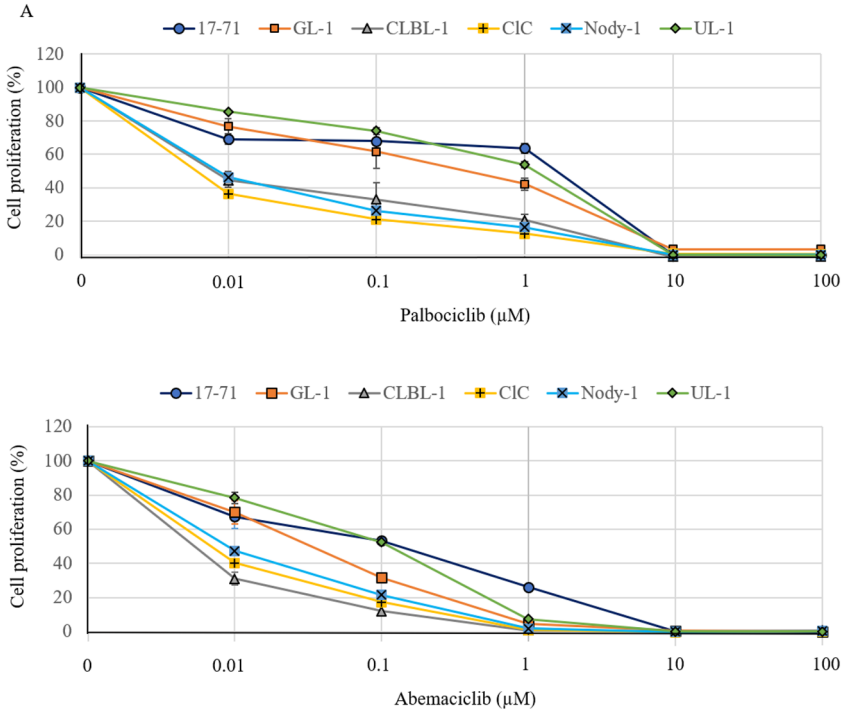
Although there are no reports using canine lymphoma/leukemia cells, IC_{50} values of 64.06 nM for P114 cells and 18.8 nM for CF41 cells has been reported in canine mammary tumors treated with palbociclib [25]. Another report found that cell proliferation decreased significantly on canine melanoma cells after being treated with palbociclib (1 µM for LMCK and OLGA cells and 2.5 µM for CMM10 cell). However, in CMM12 canine melanoma cells, cell proliferation remained relatively constant with increasing concentrations of CDK4/6i, and a significant reduction in cell proliferation was observed only at the maximum palbociclib concentration tested (10 µM) [3]. Compared with these cells, the canine lymphoma cells showed higher sensitivity to palbociclib, particularly in three of the four cell lines with low p16, as their IC_{50} values were less than 10 nM.

To examine whether palbociclib and abemaciclib treatment correlated with a decrease in pRb-P, canine lymphoma cell lines with high or low p16 levels were treated with various concentrations of palbociclib or abemaciclib (0.01, 0.1, and 1 µM) for 12 hr. The expression of pRb-P and p16 proteins was simultaneously examined by western blot analysis. Cell lysates were electrophoresed on 6% or 12% SDS polyacrylamide gels at 2 A per gel for one hr. The electrophoresed proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane (Merck Millipore, Billerica, MA, USA) at 100 V for one hr. The PVDF membrane was washed in Tris-buffered saline containing 0.1% Tween-20 (TBST), blocked for one hr with 5% non-fat milk/TBST (blocking buffer), and washed with TBST. The PVDF membrane was incubated overnight at 4°C with mouse monoclonal anti-p16 (1:500, F-8; Santa Cruz Biotechnology, Dallas, TX, USA) [19] and rabbit monoclonal anti-phospho-pRb (1:1,000, phospho-T826; Abcam, Cambridge, UK) diluted in 5% non-fat milk/TBST (antibody dilution) [7]. A mouse monoclonal anti-β-actin antibody (1:5,000 dilution of 0.5% non-fat milk/TBST, AC-15; Sigma-Aldrich) was used as the endogenous control. The PVDF membranes were washed thrice for 10 min each and incubated with HRP-conjugated goat anti-mouse or anti-rabbit IgG antibody (Santa Cruz Biotechnology) at room temperature for one hr. The membranes were then washed thrice for 10 min. Before visualization, the membranes were incubated for 5 min using the SuperSignal™ West Pico PLUS Chemiluminescent Substrate reagent (Thermo Fisher Scientific, Waltham, MA, USA). Proteins on the membranes were captured using an AMERSHAM ImageQuant 800 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Protein expression of p16 and pRb-P was quantified using ImageJ software [1]. The quantification data of ImageJ are expressed as the mean and standard deviation (SD) values and were then analyzed by one-way ANOVA and Tukey's *post-hoc* test. $P<0.05$ was set as significant statistical values.

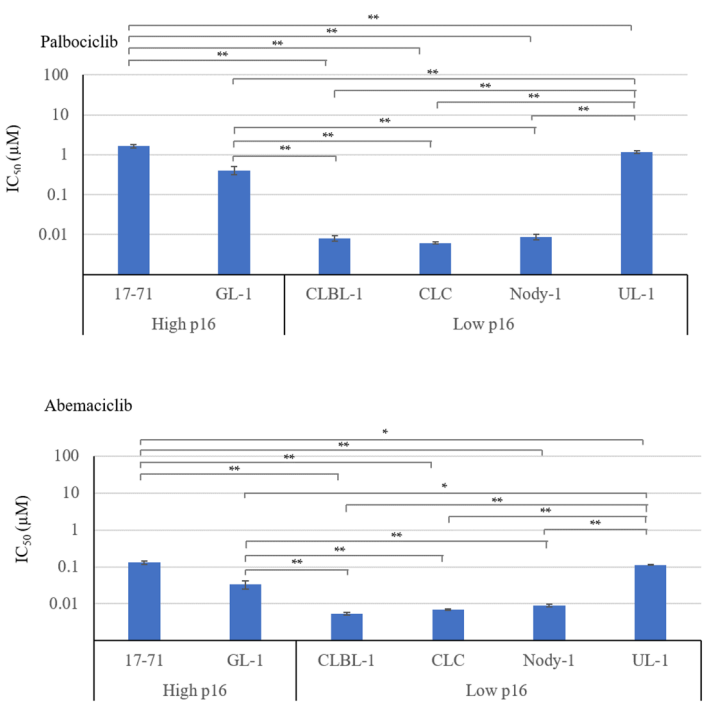
As previously shown in our study [18], p16 protein expression was observed in 17–71 and GL-1 without CDK4/6i, but not in the other cell lines (CLBL-1, CLC, Nody-1, and UL-1, Fig. 2A–F). The pRb-P was barely identified in 17–71 and GL-1 without CDK4/6i, but was clearly found in the other cell lines. Phosphorylation of pRb was reduced significantly with 0.01 µM palbociclib or abemaciclib for 12 hr, and it was reduced as the concentration was increased except in 17–71 with no expression of pRb-P with or without CDK4/6i. Our study revealed that palbociclib and abemaciclib treatment reduced pRb phosphorylation in a dose-dependent manner in canine lymphoma cells, especially in cells with low p16 and high pRb-P (CLBL-1, CLC, Nody-1, and UL-1), suggesting that CDK4/6i inhibits the phosphorylation of pRb by inhibiting CDK4/6.

We found that high p16-represented cells (17–71 and GL-1) showed low sensitivity to palbociclib and abemaciclib. A study has reported the biological sensitivity of engineered isogenic cells, indicating that high levels of p16 predict insensitivity to palbociclib [11]. In contrast, we found that one of the low p16 cell lines, UL-1, showed a higher IC_{50} similar to that of high p16 cells. We speculated

A



B



C

p16 expression	Cell lines	Palbociclib IC_{50} (μM)	Abemaciclib IC_{50} (μM)
High p16	17-71	1.6378 ± 0.1374	0.1316 ± 0.0142
	GL-1	0.4060 ± 0.0957	0.0330 ± 0.0082
Low p16	CLBL-1	0.0081 ± 0.0013	0.0053 ± 0.0005
	CLC	0.0061 ± 0.0004	0.0068 ± 0.0003
	Nody-1	0.0086 ± 0.0013	0.0089 ± 0.009
	UL-1	1.1742 ± 0.0844	0.1128 ± 0.0018

Fig. 1. Association between p16 expression and the sensitivity of cyclin-dependent kinase 4/6 inhibitors palbociclib and abemaciclib in canine lymphoma cells. (**A**) Cell proliferation (%) curve in canine lymphoma cells treated with palbociclib (upper) or abemaciclib (lower) at various concentrations. (**B**, **C**) The half-maximal inhibitory concentration (IC_{50}) values (μM) of palbociclib and abemaciclib in canine lymphoma cells with high or low p16 expressions. The proliferation and cytotoxicity assay were assessed using CCK8 assay. The data are expressed as the mean and standard deviation (SD) values of three replicates in the triplicate assay. **P*<0.05; ***P*<0.01.

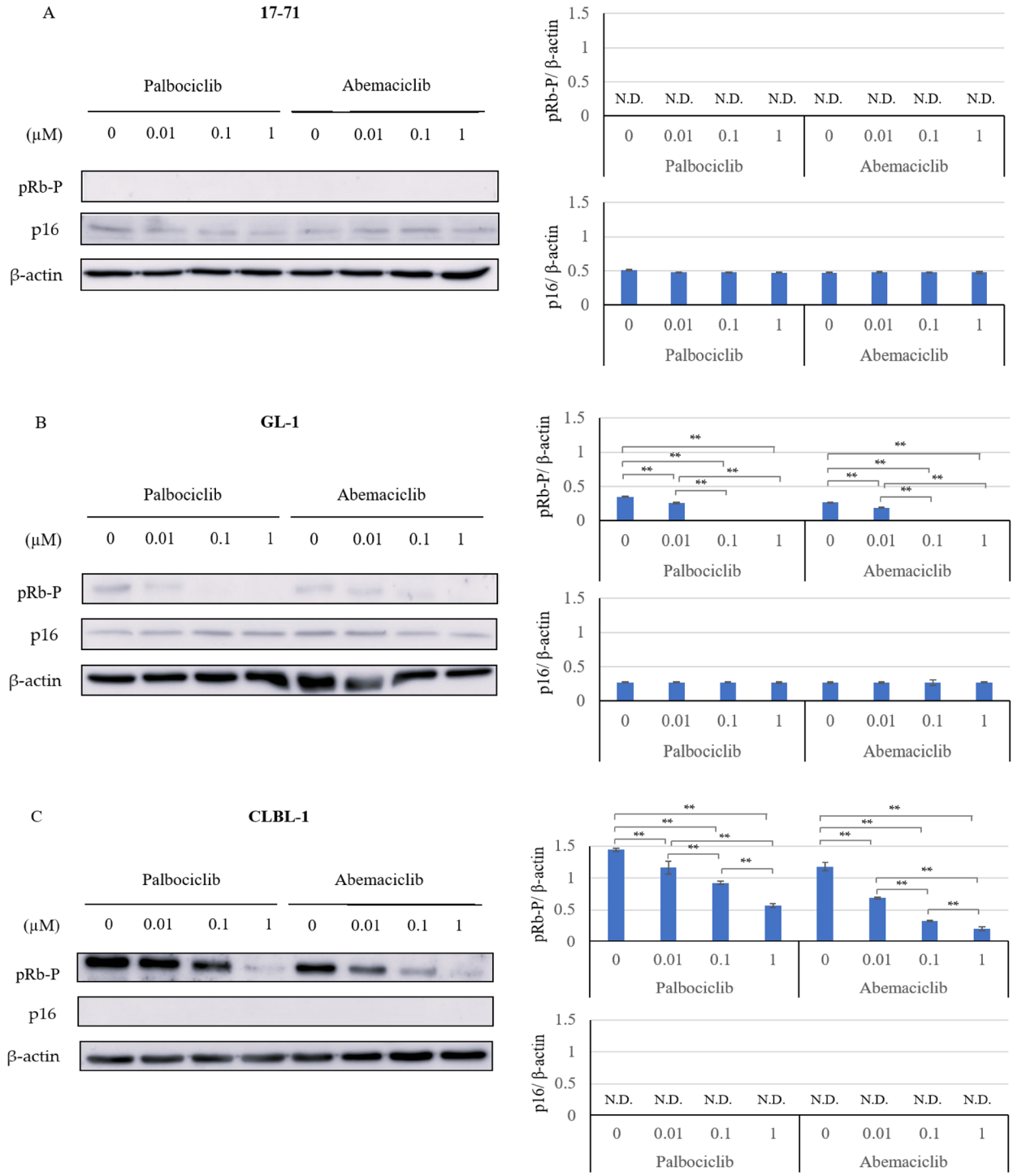


Fig. 2. Palbociclib and abemaciclib inhibit pRb phosphorylation in a dose-dependent manner. Association of palbociclib and abemaciclib treatment to the pRb phosphorylation in canine lymphoma cells with high p16 (**A**) 17-71 and (**B**) GL-1; and low p16 (**C**) CLBL-1, (**D**) CLC, (**E**) Nody-1, and (**F**) UL-1. Phosphorylated pRb (pRb-P) and p16 protein of canine lymphoma cells were assessed using western blot analysis and quantified using ImageJ software. **P*<0.05; ***P*<0.01. N.D.; not detected.

that UL-1 cells may be intrinsically resistant to CDK4/6i owing to pRb deficiency or downstream bypass [11, 13, 28]. However, as a limitation of this study, we need to further explore the mechanism(s) that mediate CDK4/6i resistance in UL-1 cells.

In conclusion, CDK4/6 inhibitors exhibit potential as new chemotherapeutic agents for canine lymphoma and high p16 protein expression may be used as a useful biomarker for resistance to CDK4/6 inhibitor therapy. Further studies are necessary to verify CDK4/6i including palbociclib and abemaciclib as potential new chemotherapeutic agents using a transplantation mouse model of canine lymphoma *in vivo*.

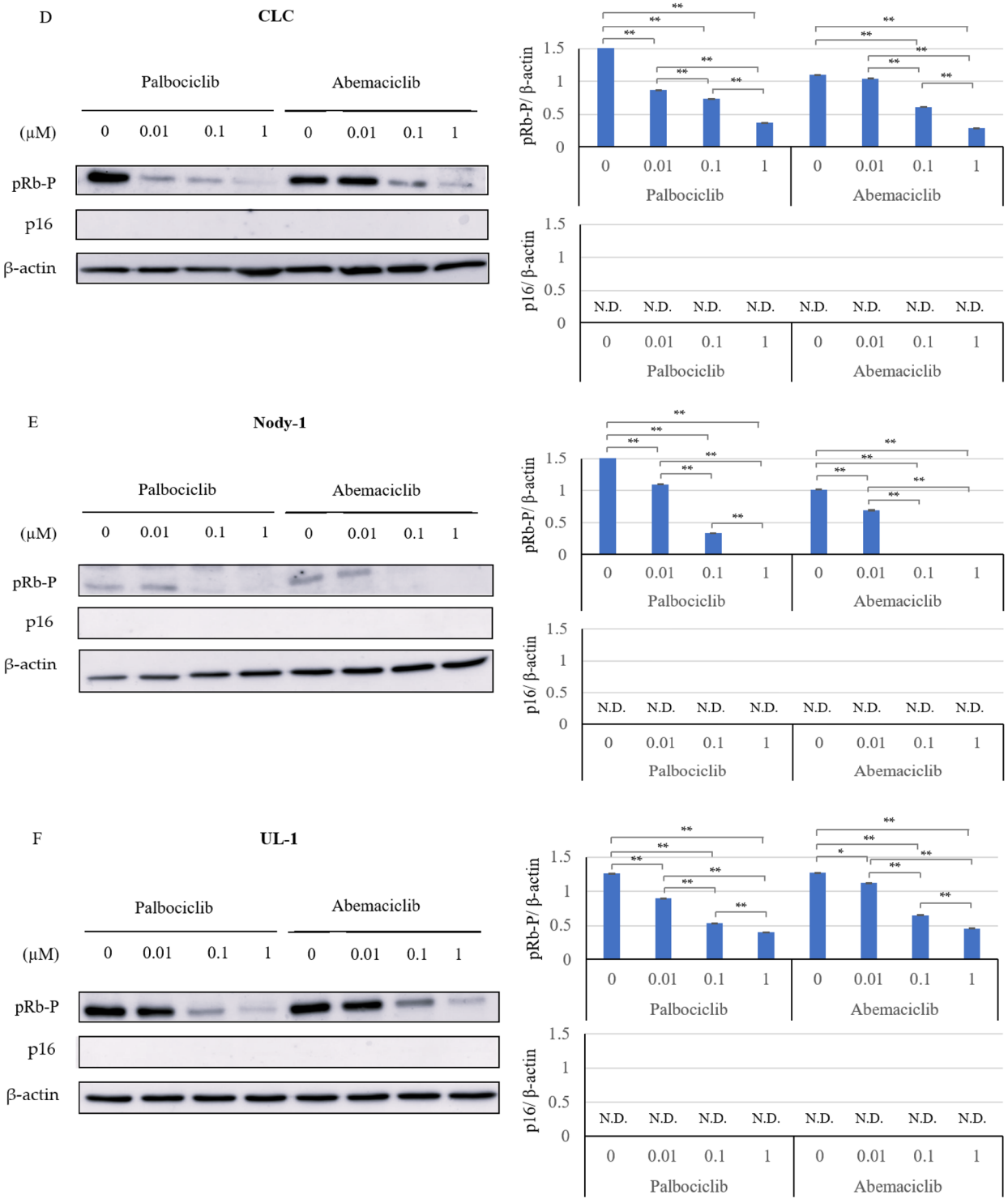


Fig. 2. Continued.

CONFLICT OF INTEREST. The authors declare that there are no conflicts of interest.

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