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Berberine slows cell growth in autosomal dominant polycystic kidney disease cells

Anna Bonon [a], Alessandra Mangolini [a], Paolo Pinton [b], Laura del Senno [a], Gianluca Aguiari [a,*]

[a] Department of Biomedical and Specialty Surgical Sciences, University of Ferrara, 44121 Ferrara, Italy
[b] Department of Morphology, Surgery and Experimental Medicine, Section of General Pathology, University of Ferrara, 44121 Ferrara, Italy

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ABSTRACT

Autosomal dominant polycystic kidney disease (ADPKD) is the most common hereditary monogenic disorder characterized by development and enlargement of kidney cysts that lead to loss of renal function. It is caused by mutations in two genes (PKD1 and PKD2) encoding for polycystin-1 and polycystin-2 proteins which regulate different signals including cAMP, mTOR and EGFR pathways. Abnormal activation of these signals following PC1 or PC2 loss of function causes an increased cell proliferation which is a typical hallmark of this disease. Despite the promising findings obtained in animal models with targeted inhibitors able to reduce cystic cell growth, currently, no specific approved therapy for ADPKD is available. Therefore, the research of new more effective molecules could be crucial for the treatment of this severe pathology. In this regard, we have studied the effect of berberine, an isoquinoline quaternary alkaloid, on cell proliferation and apoptosis in human and mouse ADPKD cystic cell lines. Berberine treatment slows cell proliferation of ADPKD cystic cells in a dose-dependent manner and at high doses (100 μg/mL) it induces cell death in cystic cells as well as in normal kidney tubule cells. However, at 10 μg/mL, berberine reduces cell growth in ADPKD cystic cells only enhancing G_0/G_1 phase of cell cycle and inhibiting ERK and p70-S6 kinases. Our results indicate that berberine shows a selected antiproliferative activity in cellular models for ADPKD, suggesting that this molecule and similar natural compounds could open new opportunities for the therapy of ADPKD patients.

1. Introduction

ADPKD is one of the most common monogenic disorders (1:1000) characterized by the progressive bilateral development and enlargement of kidney cysts that lead to end-stage renal disease (ESRD) [1]. ADPKD causes also hypertension, cardiac valve defects and intracranial aneurysms which are the major cause of death [2]. PKD1 and PKD2 gene mutations account for 85% and 15% of ADPKD cases respectively, and codify for polycystin-1 (PC1), a large membrane protein and polycystin-2 (PC2), a calcium permeable channel, respectively [2]. PC1 functions as adhesion molecule implicated in the maintenance of tubular architecture of the kidney [2,3], while PC2 is a Ca^{2+} permeable channel which may modulate calcium homeostasis [4,5]. They interact each other forming a complex able to regulate calcium channel activity in kidney cells as well as in lymphoblastoid cell lines [5–8]. This complex might regulate a series of biological features such as cell proliferation and apoptosis by modulating different signals including cAMP, mTOR and EGFR pathways [9–13]. Although several inhibitors of these ways are able to reduce kidney cysts in animal models for ADPKD, results from clinical trials are not completely satisfactory [14–18]. Moreover, there is also evidence of a reduced intracellular Ca^{2+} activity which accelerates cell proliferation in ADPKD cystic cells, resulting in kidney cyst formation, which might be reverted by the treatment with calcium channel activators [19,20]. Consistently, triptolide, a diterpene used in the traditional Chinese medicine, induces intracellular calcium release, leading to cell growth arrest in $Pkd1^{(-/-)}$ mouse cells, and reduces cellular proliferation and cyst formation in a murine model of ADPKD [21].

Here we show that the application of berberine, another product of traditional Chinese medicine, is able to reduce cell proliferation by inhibition of ERK and p70-S6 kinases in ADPKD cystic cells.

2. Material and methods

2.1. Reagents

The material for cell culture and berberine (lyophilized powder) was purchased from Sigma (Italy). Rabbit polyclonal anti-ERK,

Abbreviations: cAMP, cyclic adenosine monophosphate; mTOR, mammalian target of rapamycin; EGFR, epidermal growth factor receptor; ERK, extracellular-signal-regulated kinases.

* Corresponding author. Address: Department of Biomedical and Specialty Surgical Sciences, Via Fossato di Mortara 74, 44121 Ferrara, Italy. Fax: +39 0532 974460.
E-mail address: dsn@unife.it (G. Aguiari).

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anti-pERK, anti-S6K and anti-pS6K antibodies were obtained from Cell Signaling (Euroclone, Italy). Enhanced chemiluminescent substrates for Western blotting and HRP-conjugated goat anti-rabbit antibodies were purchased from Euroclone (Italy).

2.2. Cell proliferation and cell cycle analysis

Human normal (4/5) and cystic kidney epithelial cells (9.7 and 9.12), derived from proximal tubules of normal and polycystic kidneys [22] as well as mouse Pkd1$^{(-/-)}$ null and Pkd1$^{(+/-)}$ heterozygous kidney cystic cells [23] have been cultured in DMEM/F12 medium supplemented with 10% FBS. Cell proliferation analysis was performed plating 30,000 or 5000 cells/mL in 24- or 96-well plates, respectively. Subsequently, cells were starved for 24 h in DMEM/F12 supplemented with 0.4% BSA and treated for 24 h in DMEM-1% FBS alone or in combination with berberine (1, 10 and 100 μg/mL). Cell proliferation was calculated by direct cell counting after trypan blue staining, using a Burker chamber [24], and by a colorimetric method. The latter consists in the quantitation of formazan, a colored compound produced by the cells through the bio-reduction of tetrazolium salts added in culture medium. The amount of formazan, detectable recording the absorbance at 490 nm with a 96-well plate reader, is directly proportional to the number of living cells (CellTiter cell proliferation assay, Promega).

Cell cycle analysis was carried out by flow cytometry using the FACSCalibur Becton Dickinson Immunocytometry System [25].

The study protocol is in line with the ethical guidelines of the 1975 Declaration of Helsinki.

2.3. Apoptosis

Apoptosis was analyzed by caspase-3 assay, Hoechst method and DNA fragmentation. Caspase-3 activity was evaluated using EnzChek® caspase-3 Assay Kit (Invitrogen). After treatment with 1% FBS alone or in combination with 10 and 100 μg/mL of berberine for 24 h, cells were harvested, lysed and centrifuged according to the manufacturer's instructions. 50 μL aliquots of supernatant were incubated with 50 μL of 2× substrate working solution containing 5 mM Z-DEVD-R110. Fluorescence was measured every minute for 90 min at 520 nm by a fluorimeter (VICTOR3 1420 Multilabel Counter, PerkinElmer). Values were normalized with the protein content by the Bradford method.

Apoptotic nuclei were detected by Hoechst method in cells cultured in 0.4% BSA alone or in combination with 10 and 100 μg/mL of berberine for 24 h. The presence of apoptotic cells was determined by fluorescence after cell staining with Hoechst 33258 (10 μg/mL). Images were acquired through a Zeiss Axiovert 200 fluorescence microscope, equipped with a back-illuminated CCD camera (Roper Scientific, Tucson, AZ).

DNA fragmentation was evaluated after DNA isolation by phenol/chloroform extraction. Ladder DNA was separated by electrophoresis in a 1.5% agarose gel and visualized through an UV transilluminator Bio RAD (Italy) after staining with ethidium bromide.

2.4. Western blotting

Berberine treated and untreated cells were lysed in a 1% Triton X-100 solution and processed for immunoblots as already described [6]. Blocked membranes were probed overnight at 4 °C with the primary antibody and then for 2 h with the secondary antibody. Finally, proteins were visualized using the chemiluminescence system. Band intensity was detected by X-ray film scanning with a imaging densitometer (BIO-RAD, Italy). Quantitative phosphorylation was calculated as ratio between phosphorylated and un-phosphorylated protein [10].

2.5. Statistical analysis

Analysis of data was performed using Student's *t* test (unpaired analysis). Differences are considered significant at a value of $p < 0.05$. All data are reported as mean ± SD (standard deviation) of at least three independent experiments in duplicate.

3. Results

3.1. Berberine slows cell proliferation by cell accumulation in G_0/G_1 phase of cell cycle

To verify if berberine may inhibit cell proliferation of ADPKD cells, we have treated normal (4/5) and cystic (9.7 and 9.12) cells with different doses of berberine (1, 10 and 100 μg/mL). The treatment with 10 μg/mL of berberine causes a significant reduction of cell proliferation in 9.7 and 9.12 cells, but not in normal 4/5 cells (Fig. 1A). At 100 μg/mL the effect of this molecule is stronger but also affects control cells (Fig. 1A). At this dose, the reduction in cell number is associated with an overall increase of dead cells, not observed at lower doses (Fig. 1A). The decrease in cell proliferation is still maintained after 48 and 72 h of culture with 10 μg/mL of berberine in 9.7 and 9.12 cystic cells only. As observed for 24 h of culture, berberine at 100 μg/mL affects also 4/5 control cells (Fig. 1B and C) and induces cell death in all cell types (data not shown). Consistently, the reduction of cell growth after 10 μg/mL of berberine is also shown in mouse Pkd1$^{(-/-)}$ null cells as well as in Pkd1$^{(+/-)}$ heterozygous cells which are both PKD1 haploinsufficient (Fig. 1D). In these cells, the treatment with 100 μg/mL of berberine still induces cell death, although not as strong as in human cells (Fig. 1D and A, respectively). By a colorimetric method, the reduction of cell growth after berberine treatment (10 and 100 μg/mL) in both human and mouse cystic cells has been also confirmed (Fig. 2A and B). No significant changes after treatment with 1 μg/mL of berberine are observed (Fig. 1A, D and Fig. 2B).

The effect of berberine on cell proliferation has been also evaluated by cell cycle analysis in human normal and ADPKD cells. The treatment with 1 μg/mL of berberine does not modify the cell cycle (Fig. 2C), but at the dose of 10 μg/mL a significant increase of G_0/G_1 phase in 9.7 and 9.12 cystic cells compared with untreated cells is observed (Fig. 2C). Conversely, an higher dosage of berberine (100 μg/mL) causes a strong rise of G_0/G_1 phase in all cell lines, with the appearance of an apoptotic pre-G_1 peak in 4/5 control cells (arrow in Fig. 2C).

In the light of these findings, 10 μg/mL of berberine seems to be the optimal dose capable to reduce cell growth in ADPKD cells without inducing cell death.

3.2. Berberine decreases cell proliferation through the reduction of ERK and p70-S6 kinase activity

Since berberine inhibits the metastatic potential of melanoma cells through a decrease in ERK activity [26], we have investigated whether the berberine-induced reduction of cell proliferation in ADPKD cystic cells occurs through the inhibition of ERK kinase. As expected, in normal conditions (1% FBS), ERK activity is higher in cystic than in control cells (Fig. 3A). After treatment with 10 μg/mL of berberine a statistically significant reduction in ERK phosphorylation in 9.7 and 9.12 cystic cells only is observed (Fig. 3A). Moreover, berberine is able to reduce the activity of p70-S6 kinase, a downstream effector of mTOR in 9.7 and 9.12 cystic cells (Fig. 3B). Data are also confirmed in mouse Pkd cystic cells (data not shown).

These findings suggest that this molecule may reduce cell proliferation in ADPKD cystic cells by inhibiting the activity of ERK and p70-S6 kinases.

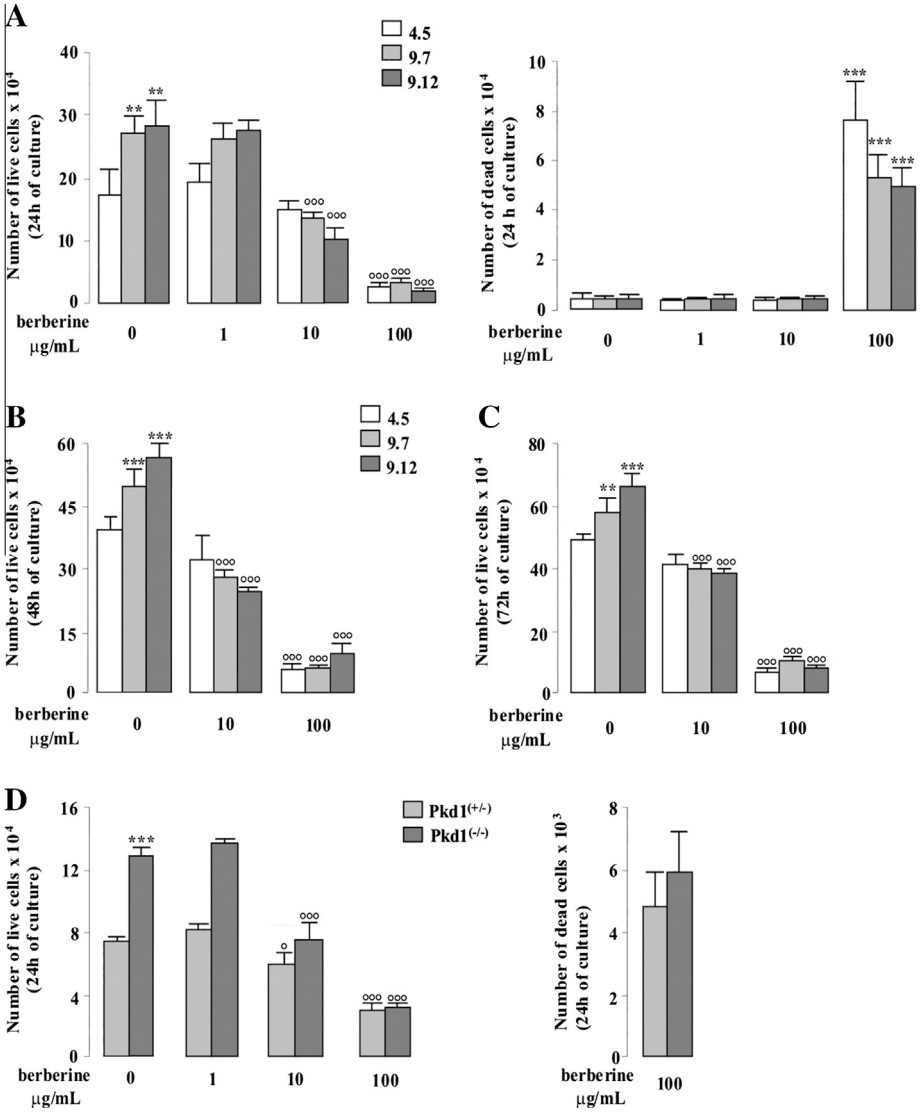

Fig. 1. Berberine reduces cell proliferation in ADPKD cystic cells. (A) Live (left) and dead cells (right), were directly counted in a Burker chamber after treatment with DMEM/F12 1% FBS or DMEM/F12 1% FBS plus 1, 10 and 100 μg/mL of berberine for 24 h. The number of live cells in 1% FBS is 176,000 ± 46,667 for 4/5, 272,222 ± 28,350 for 9.7 and 283,333 ± 52,387 for 9.12 ($^{**}p < 0.01$ for 9.12 and 9.7 vs. 4/5). After the treatment with 10 μg/mL of berberine the cell number for 9.7 and 9.12 is reduced to 136,667 ± 10,000 and 96,666 ± 6736, respectively ($^{°°°}p < 0.001$, for 9.7 and 9.12 treated vs. untreated cells). Berberine at 100 μg/mL strongly reduces the cell number of both normal 4/5 and cystic 9.7 and 9.12 cells ($^{°°°}p < 0.001$, treated vs. untreated cells). This amount of drug increases the number of trypan blue-positive dead cells. ($^{***}p < 0.001$ for treated vs. untreated cells). (B) Cell counting after 48 h of culture in 1% FBS or in 1% FBS plus 10 and 100 μg/mL of berberine. ($^{***}p < 0.001$ for 9.7 and 9.12 cells vs. 4/5 cells cultured for 48 h without berberine; $^{°°°}p < 0.001$ for cells treated with 10 and 100 μg/mL of berberine vs. untreated cells). (C) Cell number after 72 h of culture in the same conditions of point B. The statistical significance is: $^{**}p < 0.01$ and $^{***}p < 0.001$ for 9.7 and 9.12 cells, respectively vs. 4/5 control cells in absence of berberine; $^{°°°}p < 0.001$ for cells treated with 10 and 100 μg/mL of berberine vs. untreated cells. (D) Berberine at doses of 10 and 100 μg/mL decreases the number of live cells (left) of both $Pkd1^{(-/-)}$ null and $Pkd^{(+/-)}$ heterozygous mouse cystic cell lines. The statistical significance is: $^{***}p < 0.001$ for $Pkd1^{(-/-)}$ null vs. $Pkd1^{(+/-)}$ heterozygous cells cultured in absence of berberine; $^{°}p < 0.05$ and $^{°°°}p < 0.001$ for $Pkd1^{(+/-)}$ cells treated with 10 and 100 μg/mL of berberine, respectively vs. untreated cells (light grey bars); $^{°°°}p < 0.001$ for $Pkd1^{(-/-)}$ cells treated with 10 and 100 μg/mL of berberine vs. untreated cells (dark grey bars). No trypan blue positive cells are observed after treatment with 1 and 10 μg/mL of berberine in both $Pkd1^{(-/-)}$ and $Pkd1^{(+/-)}$ mouse cell lines, while an higher dose of berberine (100 μg/mL) causes an increase of dead cells (right, ~4–6 × 10^3 cells/mL).

3.3. Higher doses of berberine cause apoptosis in both normal and ADPKD kidney cells

As previously reported, a strong increase of G_0/G_1 phase with the occurrence of an apoptotic pre-G_1 peak after cell treatment with 100 μg/mL of berberine has been observed (Fig. 2C). To confirm this data, we have analyzed the activity of caspase-3 in cells treated with 100 μg/mL of berberine. This treatment causes a marked increase of caspase-3 activity in 4/5 cells as well as in 9.7 and 9.12 ADPKD cells with respect to untreated cells (Fig. 3C). At 10 μg/mL berberine does not significantly affect the activity of caspase-3 (Fig. 1 supplementary data). Consistently, the presence of apoptotic nuclei after Hoechst 33258 staining only in cells treated with 100 μg/mL of berberine is shown (Fig. 4A). Moreover, this dose of berberine also causes DNA fragmentation in both normal (4/5) and cystic (9.7 and 9.12) cells, not observable at lower doses (Fig. 4B). Taken together, these findings suggest that berberine at low dosage is able to reduce ADPKD cell proliferation without inducing apoptosis.

4. Discussion

Given the cell proliferation associated with cyst growth in ADPKD cells [27], an attractive therapeutic strategy to ameliorate clinical picture of ADPKD patients remains the use of antiproliferative molecules to break down the hyperproliferative nature of the disease. However, many compounds that inhibit signaling

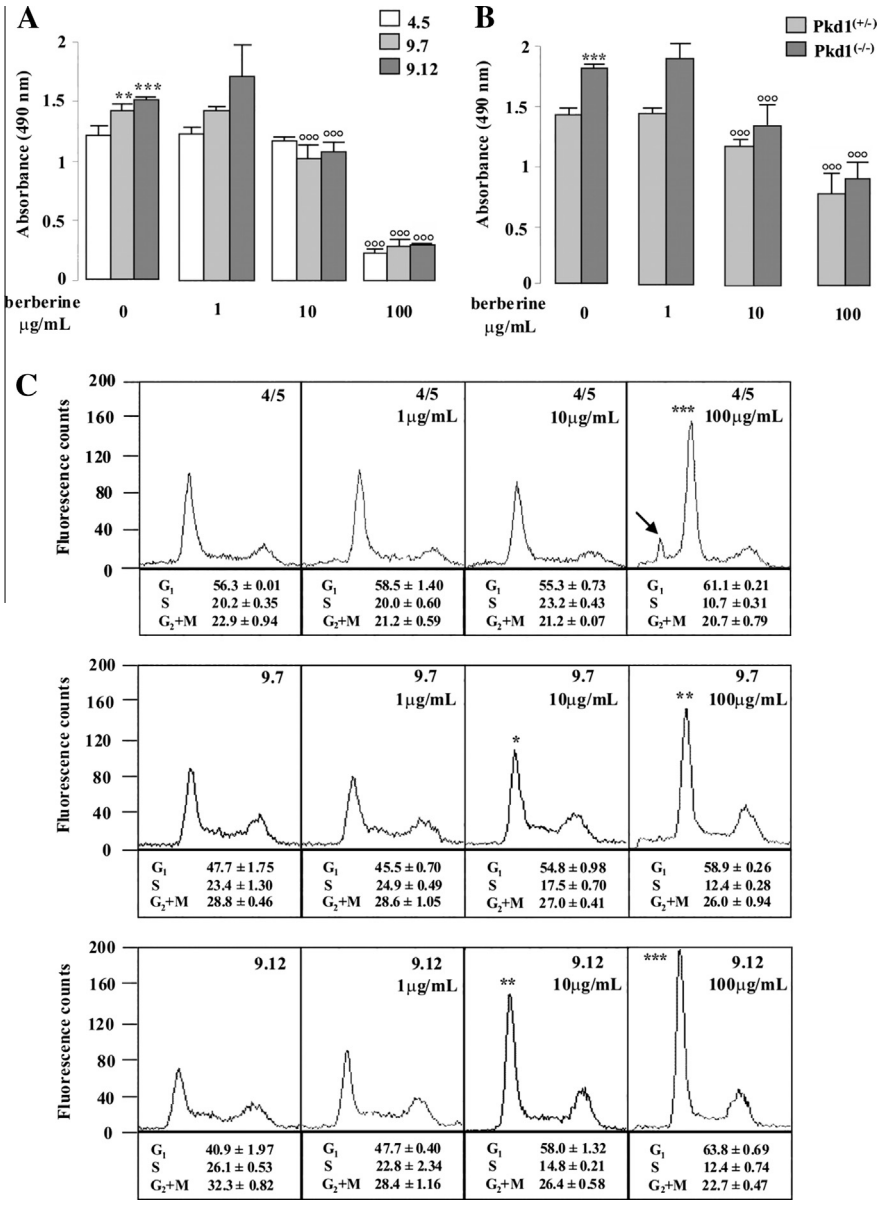

Fig. 2. Berberine-induced inhibition of cell growth occurs through the accumulation of cells in G_0/G_1 phase of cell cycle. (A) Cell proliferation is evaluated by CellTiter protocol (see methods) in human kidney cell lines. Cells, seeded at 5000/well in a 96-well plate, are starved for 24 h in 0.4% BSA and cultured for further 24 h with different doses of berberine. In absence of berberine the number of living (9.7 and 9.12) cystic cells is higher than (4/5) control cells ($^{**}p < 0.01$ and $^{***}p < 0.001$ for 9.7 and 9.12 cells, respectively vs. 4/5 cells). The treatment with 10 μg/mL of berberine causes a reduction of cell proliferation in 9.7 and 9.12 cells compared with untreated ($^{\circ\circ\circ}p < 0.001$). Higher doses of berberine (100 μg/mL) induce a strong inhibition of cell growth in all cell types ($^{\circ\circ\circ}p < 0.001$). (B) Cell proliferation measured by CellTiter method, in the same conditions of point A, in mouse $Pkd1^{(-/-)}$ null and $Pkd1^{(+/-)}$ heterozygous cell lines. The statistical significance is: $^{***}p < 0.01$ for $Pkd1^{(-/-)}$ vs. $Pkd1^{(+/-)}$ cells cultured in 1% FBS; $^{\circ\circ\circ}p < 0.001$ for cells treated with 10 and 100 μg/mL of berberine vs. untreated. (C) In human cystic cells (9.7 and 9.12) treated with 10 μg/mL of berberine is observed an increase in G_0/G_1 phase of cell cycle compared with untreated cells ($^{*}p < 0.05$ in 9.7 and $^{**}p < 0.01$ in 9.12 cells). At the dose of 100 μg/mL, berberine causes a strong increase of G_0/G_1 phase in 4/5 control cells ($^{***}p < 0.001$) as well as in 9.7 ($^{**}p < 0.01$) and 9.12 ($^{***}p < 0.001$) cystic cells as compared to untreated cells. In 4/5 control cells, a pre-G_1 peak (indicated by the arrow), showing the presence of apoptotic cells, also appears.

pathways altered in ADPKD, in particular mTOR inhibitors, currently, did not give the expected results [14–16]. Therefore, the discovery of new molecules could be crucial for the treatment of this pathology. In this regards, the observation that berberine, a natural isoquinoline alkaloid, shows antiproliferative properties in different cancer cells [28], prompt us to evaluate the antiproliferative effects of berberine in two "*in vitro*" cellular models for ADPKD; one derived from human polycystic kidneys (9.7 and 9.12 cells) and the second isolated from kidney cysts of polycystic mice ($Pkd1^{(-/-)}$ and $Pkd1^{(+/-)}$ cells). Here we demonstrate that the treatment with 10 μg/mL of berberine causes a marked reduction of cell proliferation in both human and mouse ADPKD cystic cells (Fig. 1 and 2A–B). The decrease of cell growth induced by 10 μg/mL of berberine is associated with an increased G_0/G_1 phase of cell cycle in cystic cells (Fig. 2C), as already observed in cancer cells [28]. Since the reduction of cell proliferation by the treatment with 10 μg/mL of berberine is associated with a strong inhibition ERK phosphorylation in ADPKD cystic cells (Fig. 3A), we suggest that the berberine induced-reduction of cell proliferation of ADPKD cystic cells may be mediated by the inhibition of ERK signaling. It is, in fact, known that in ADPKD epithelial cells the sequential phosphorylation of PKA, B-Raf and MAPK kinases activates ERK

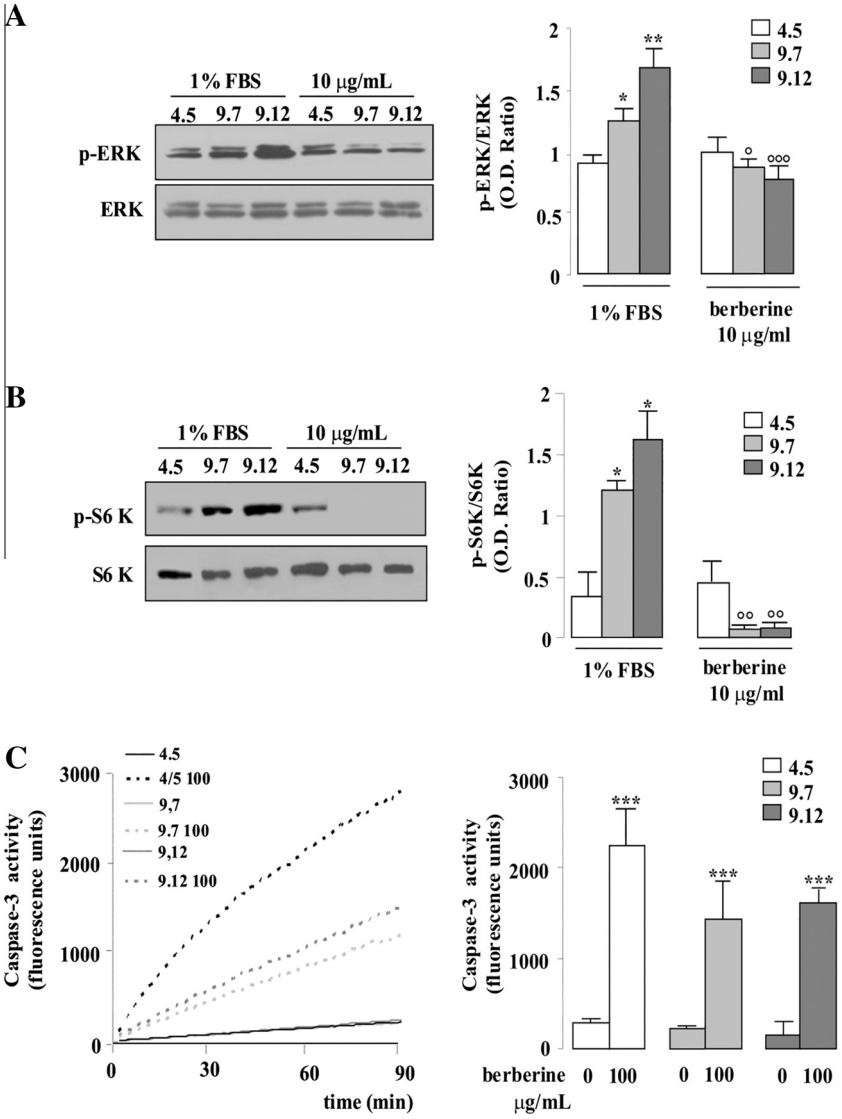

Fig. 3. Low dosage of berberine (10 μg/mL) reduces the activity of ERK and p70-S6 kinases, but at higher doses (100 μg/mL) induces apoptosis by caspase-3 activation. (A) The activity of ERK kinases was analyzed by western blotting in cells cultured in DMEM/F12 1% FBS alone or in combination with 10 μg/mL of berberine. In 1% FBS, ERK activity was higher in 9.7 (1.25 ± 0.15) and 9.12 (1.68 ± 0.15) than in 4/5 cells (0.9 ± 0.07); $^{*}p < 0.05$ and $^{**}p < 0.01$ for 9.7 and 9.12 vs. 4/5 cells, respectively. The treatment with 10 μg/mL of berberine reduces the activity of ERK kinases to 0.87 ± 0.05 in 9.7 and 0.76 ± 0.1 in 9.12 cells ($^{\circ}p < 0.05$ for 9.7 and $^{\circ\circ\circ}p < 0.001$ for 9.12 treated vs. untreated, respectively). Data were calculated on three different experiments in duplicate. (B) p70-S6 Kinase activity calculated by western blotting after cell treatment in presence and absence of berberine (10 μg/mL). In 1% FBS the activity of p70-S6K is higher in 9.7 (1.21 ± 0.07) and 9.12 (1.62 ± 0.22) than in 4/5 cells (0.32 ± 0.19); $^{*}p < 0.05$ in 9.7 and 9.12 vs. 4/5 cells. The treatment with 10 μg/mL of berberine strongly reduces p70-S6K activity in 9.7 (0.07 ± 0.02) and 9.12 (0.058 ± 0.03) cystic cells ($^{\circ\circ}p < 0.01$ for 9.7 and 9.12 cells treated with 10 μg/mL of berberine vs. untreated). Data were calculated on two different experiments in duplicate. (C) Cells cultured for 24 h in DMEM/F12 1% FBS alone or in combination with 100 μg/mL of berberine were analyzed for the activity of caspase-3 as described in method section. The activity of caspase-3 in both 4/5 control and in 9.7 and 9.12 cystic cells treated with 100 μg/mL of berberine is increased compared with untreated cells ($^{***}p < 0.001$ for treated vs. untreated cells).

signaling that stimulates cell proliferation [9]. It has been also reported that in multiple tumor cells, berberine shows a suppressive effect on cell invasion inhibiting many important signaling pathways including ERK and p38 MAPK kinases [26]. Berberine-dependent inhibition of ADPKD cell growth should be also associated with a reduction of mTOR signaling which is modulated by ERK kinases in ADPKD cystic cells [18,29]. Consistently, berberine treatment reduces the activity of p70-S6 kinase, a downstream effector of mTOR in ADPKD cystic cells (Fig. 3B), thus, this compound might affect the mTOR cascade that plays an important role in ADPKD cyst development [30]. However, the treatment with 100 μg/mL of berberine induces a strong cell death in both normal and cystic cells (Fig. 1A and D), as well as a marked cell accumulation in G0/G1 phase of the cell cycle (Fig. 2C). In addition, in 4/5 control cells appear an apoptotic pre-G1 peak (arrow in Fig. 2C), also observed in oral squamous carcinoma cells [31]. In these cancer cells, higher doses of berberine induce apoptosis through a reduction of the mitochondrial membrane potential associated with the release of cytochrome c and activation of caspase-3 [31]. Consistently, the treatment of cells with 100 μg/mL of berberine induces an increase of caspase-3 activity (Fig. 3C), the formation of apoptotic nuclei (Fig. 4A) and DNA fragmentation (Fig. 4B) in both control and cystic cells. On the other hand, it is known that high doses of berberine may cause cytotoxic effects non only in cancer cells but also in normal cell lines [32].

In conclusion, our findings suggest that berberine, at appropriate doses, slows cell proliferation in two different cellular models for the autosomal dominant polycystic kidney disease, making this

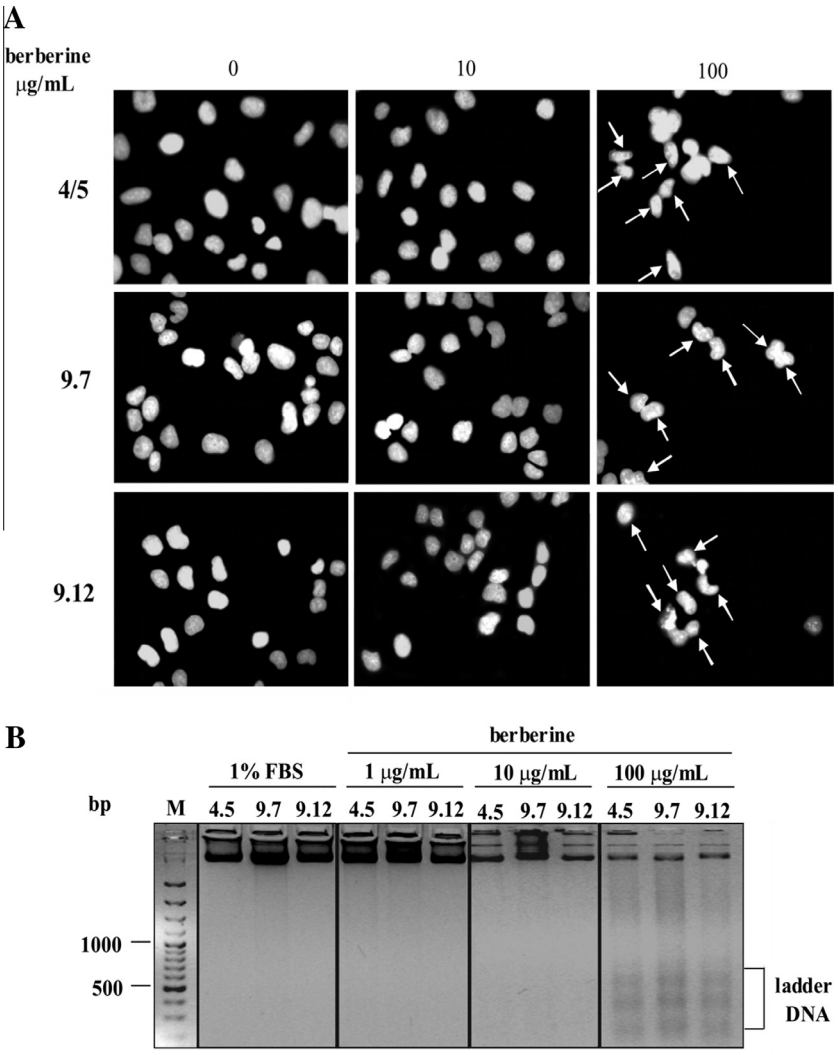

Fig. 4. The treatment with 100 μg/mL of berberine induces apoptotic bodies formation and DNA fragmentation. (A) Apoptotic cells were detected by nuclei staining with Hoechst-33258 in cells cultured in absence or presence of berberine (10 and 100 μg/mL). Images were acquired with 40x magnification by a florescence microscope equipped with a CCD camera. The treatment of control and cystic cells with 100 μg/mL of berberine simulates the formation of apoptotic bodies (see arrows). (B) DNA was extracted by cells cultured in presence of 1% FBS alone or in combination with 1, 10 and 100 μg/mL of berberine. DNA electrophoresis on 1.5% agarose gel shows the fragmentation of DNA in both normal (4/5) and cystic (9.7 and 9.12) cells treated with 100 μg/mL of berberine. No DNA fragmentation in the other culture conditions are observed.

molecule an additional candidate for a future pharmacological therapy of ADPKD.

Conflict of interest

None.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.10.076.

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