Dear Author,

Please, note that changes made to the HTML content will be added to the article before publication, but are not reflected in this PDF.

Note also that this file should not be used for submitting corrections.

AUTHOR QUERY FORM

ELSEVIER	**Journal: CLS** **Article Number: 8332**	**Please e-mail or fax your responses and any corrections to:** **Mahalakshmi Gunasekaran** **E-mail: Corrections.ESCH@elsevier.spitech.com** **Fax: +1 619 699 6721**

Dear Author,

Please check your proof carefully and mark all corrections at the appropriate place in the proof (e.g., by using on-screen annotation in the PDF file) or compile them in a separate list. Note: if you opt to annotate the file with software other than Adobe Reader then please also highlight the appropriate place in the PDF file. To ensure fast publication of your paper please return your corrections within 48 hours.

For correction or revision of any artwork, please consult http://www.elsevier.com/artworkinstructions.

We were unable to process your file(s) fully electronically and have proceeded by

☐ Scanning (parts of) your article ☐ Rekeying (parts of) your article ☐ Scanning the artwork

Any queries or remarks that have arisen during the processing of your manuscript are listed below and highlighted by flags in the proof. Click on the 'Q' link to go to the location in the proof.

Location in article	Query / Remark: click on the Q link to go Please insert your reply or correction at the corresponding line in the proof
Q1	Please confirm that given names and surnames have been identified correctly. Please check this box if you have no corrections to make to the PDF file. ☐

Thank you for your assistance.

Cellular Signalling xxx (2014) xxx

Contents lists available at ScienceDirect

Cellular Signalling

journal homepage: www.elsevier.com/locate/cellsig

Highlights

Cellular Signalling xxx (2014) xxx – xxx

Inhibition of protein phosphatase-1 and -2A decreases the chemosensitivity of leukemic cells to chemotherapeutic drugs

Dóra Dedinszki [a], Andrea Kiss [a], László Márkász [b], Adrienn Márton [c], Emese Tóth [a], László Székely [d], Ferenc Erdődi [a,*]

[a] Department of Medical Chemistry, Faculty of Medicine, University of Debrecen, Debrecen, Hungary
[b] Department of Women's and Children's Health, Uppsala University, Uppsala, Sweden
[c] Department of Internal Medicine, Faculty of Medicine, University of Debrecen, Debrecen, Hungary
[d] Department of Microbiology, Tumor and Cell Biology (MTC), Karolinska Institute, Stockholm, Sweden

- Protein phosphatase-1 (PP1) and -2A (PP2A) mediate chemosensitivity of leukemic cells.
- Phosphatase inhibition attenuates chemotherapeutic drug-induced apoptotic cell death.
- The phosphorylation level of retinoblastoma protein (pRb) may control chemosensitivity.
- PP1 and its phosphorylatable inhibitory protein are involved in pRb dephosphorylation
- PP2A influences dephosphorylation of PP1 inhibitory proteins.

http://dx.doi.org/10.1016/j.cellsig.2014.11.021
0898-6568/

Cellular Signalling xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Cellular Signalling

journal homepage: www.elsevier.com/locate/cellsig

Inhibition of protein phosphatase-1 and -2A decreases the chemosensitivity of leukemic cells to chemotherapeutic drugs

Dóra Dedinszki [a], Andrea Kiss [a], László Márkász [b], Adrienn Márton [c], Emese Tóth [a], László Székely [d], Ferenc Erdődi [a,*]

[a] Department of Medical Chemistry, Faculty of Medicine, University of Debrecen, Debrecen, Hungary
[b] Department of Women's and Children's Health, Uppsala University, Uppsala, Sweden
[c] Department of Internal Medicine, Faculty of Medicine, University of Debrecen, Debrecen, Hungary
[d] Department of Microbiology, Tumor and Cell Biology (MTC), Karolinska Institute, Stockholm, Sweden

ARTICLE INFO

Article history:
Received 21 October 2014
Accepted 21 November 2014
Available online xxxx

Keywords:
THP-1
KG-1 leukemic cells
Daunorubicin
Protein phosphatase-1 and -2A
Retinoblastoma protein
Kinase-enhanced protein inhibitor (KEPI) of protein phosphatase-1

ABSTRACT

The phosphorylation of key proteins balanced by protein kinases and phosphatases are implicated in the regulation of cell cycle and apoptosis of malignant cells and influences anticancer drug actions. The efficacy of daunorubicin (DNR) in suppression of leukemic cell survival was investigated in the presence of tautomycin (TM) and calyculin A (CLA), specific membrane permeable inhibitors of protein phosphatase-1 (PP1) and -2A (PP2A), respectively. CLA (50 nM) or TM (1 μM) suppressed viability of THP-1 and KG-1 myeloid leukemia cell lines to moderate extents; however, they significantly increased survival upon DNR-induced cell death. CLA increased the phosphorylation level of Erk1/2 and PKB/Akt kinases, the retinoblastoma protein (pRb), decreased caspase-3 activation by DNR and increased the phosphorylation level of the inhibitory sites (Thr696 and Thr853) in the myosin phosphatase (MP) target subunit (MYPT1) as well as in a 25 kDa kinase-enhanced phosphatase inhibitor (KEPI)-like protein. TM induced enhanced phosphorylation of pRb only, suggesting that this event may be a common factor upon CLA-induced PP2A and TM-induced PP1 inhibitory influences on cell survival. Silencing PP1 by siRNA in HeLa cells, or overexpression of Flag-KEPI in MCF-7 cells coupled with inducing its phosphorylation by PMA or CLA, resulted in increased phosphorylation of pRb. Our results indicate that PP1 directly dephosphorylates pRb, while PP2A might have an indirect influence via mediating the phosphorylation level of PP1 inhibitory proteins. These data imply the importance of PP1 inhibitory proteins in controlling the phosphorylation state of key proteins and regulating drug sensitivity and apoptosis in leukemic cells.

1. Introduction

Chemotherapy is a common way to treat hematological malignancies to eliminate malignant cells from the body by inducing cell death. Anticancer drugs are known to induce apoptosis by activating proapoptotic regulators and at the same time suppressing survival factors [1]. Several signaling pathways may influence the efficacy of chemotherapeutic drugs and it often includes the modification of the activity of pro- and anti-apoptotic factors by Ser/Thr-specific phosphorylation/dephosphorylation [2,3]. This recognition has directed attention to the regulatory functions of protein kinases and phosphatases as their actual activity ratio determines the phosphorylation state of key proteins in cell survival. It is well established that the elements of certain protein kinase cascades, such as p42/44Erk1/2 and some other MAP-kinases [4,5] as well as Akt/PKB kinase [6], are important determinants of malignant cell apoptosis. They can exert protective influence against the action of anticancer drugs increasing survival, or, on the contrary, the activation of some MAP-kinase types might contribute to the destructive cellular effects.

Protein phosphatase-1 (PP1) and -2A (PP2A), are two major types of the phospho-Ser/Thr-specific protein phosphatases and they also play essential roles in the regulation of cell death or survival [7,8]. It has been shown that induction of cell death by chemotherapeutic agents is often coupled with phosphatase activation [9,10], while on the

Abbreviations: BSA, bovine serum albumin; CHAPS, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate; CLA, calyculin A; CPI-17, C-kinase phosphatase-1 inhibitory protein of 17 kDa; DMSO, dimethyl sulfoxide; DNR, daunorubicin; EDTA, ethylenediaminetetraacetic acid; FBS, fetal bovine serum; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; His-I-2, hexahistidine-tagged recombinant inhibitor-2; KEPI, kinase-enhanced phosphatase inhibitor; LDH, lactate dehydrogenase; MP, myosin phosphatase; MYPT1, myosin phosphatase target subunit; OA, okadaic acid; PBS, phosphate-buffered saline; PI, propidium iodide; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; ^{32}P-MLC20, ^{32}P-labeled 20 kDa light chain of turkey gizzard myosin; PMSF, phenylmethanesulfonylfluoride; PNUTS, protein phosphatase 1 nuclear targeting subunit; PP1, protein phosphatase-1; PP1c, protein phosphatase-1 catalytic subunit; PP2A, protein phosphatase-2A; pRb, retinoblastoma protein; SDS, Na-dodecyl sulfate; SDS–PAGE, SDS–polyacrylamide gel electrophoresis; siRNA, small interfering RNA; TBS, Tris-buffered saline; TBST, TBS containing 0.5% Tween-20; TM, tautomycin

* Corresponding author at: Department of Medical Chemistry, Faculty of Medicine, University of Debrecen, Egyetem tér 1, H-4032 Debrecen, Hungary. Tel.: +36 52 412345; fax: +36 52 412566.
E-mail address: erdodi@med.unideb.hu (F. Erdődi).

http://dx.doi.org/10.1016/j.cellsig.2014.11.021
0898-6568/

other hand phosphatase inhibition by cell-permeable inhibitory toxins such as calyculin A (CLA), tautomycin (TM) or okadaic acid (OA) could also decrease cell viability [11–13]. Intriguingly, when the drug and phosphatase inhibitory treatments are coupled, then phosphatase inhibition generally increases the survival of leukemic cells against several drugs implicating the protein phosphatases in the regulation of chemosensitivity of these cells [11,13–15]. However, the molecular mechanisms for the action of protein phosphatases have not been characterized in details. It is believed that PP2A inhibition plays a role in the suppression of apoptosis by diminishing Bax translocation to mitochondria, while PP1 inhibition is thought to be involved in decreasing of CD95/Fas death receptor induced apoptosis [11].

The regulation of cell cycle in malignancy is also an important issue in the uncontrolled growth of cancer cells [16,17]. Retinoblastoma protein (pRb), the product of a tumor suppressor gene, is an essential element of the checkpoint in G1/S transition of cell cycle, and its deletion or mutations are often identified in a variety of different cancers [18]. Hypophosphorylated pRb binds E2F family transcription factors strongly, thereby suppressing transcription of genes necessary for cell cycle progression. Moreover, pRb in association with E2F1 transcription factor can bind to transcriptionally active proapoptotic promoters and this is required to maximal apoptotic effects [19]. The phosphorylation of pRb by distinct cyclin/CDK complexes leads to dissociation of E2F1 allowing progression through G1/S transition and synthesis of the genes in S phase [20]. pRb is phosphorylated at more than 10 Ser/Thr residues, and phosphorylation and communication between several of these sites, such as Thr373, Ser608, Ser612, Thr821 and Thr826, might be implicated in inducing structural changes in pRb which promote possible dissociation of E2F1 [18]. However, among these sites, Thr821 or Thr826 are with special importance since phosphorylation of either residue may result in disruption of the interaction of pRb with interacting partners [20]. While the identity of the protein kinases that phosphorylate the Ser/Thr residues in pRb are well established, the protein phosphatases involved in the dephosphorylation processes are described less in details, although many studies have been carried out to date [20]. These results substantiate that the phospho-Ser/Thr sites in pRb are dephosphorylated by PP1 or PP2A, or both enzymes.

In our previous study, we showed that the C-terminal phosphorylation sites of pRb phosphorylated by cyclinE/CDK2 and cyclinD/CDK4 were preferentially dephosphorylated by PP1, and that myosin phosphatase (MP) in which PP1 catalytic subunit (PP1c) is complexed with myosin phosphatase target subunit 1 (MYPT1) might be one of the PP1 holoenzyme acting on phospho-pRb in THP-1 leukemic cells [13]. In accordance with the above data, increased inhibitory phosphorylation of MP in MYPT1 was correlated with elevated pRb phosphorylation and increased chemosensitivity of THP-1 cells to daunorubicin (DNR) treatment. It was also proven that there is a competition between pRb and MYPT1 for binding of PP1c since both proteins include an RVxF like PP1c-binding motif. With respect to the latter it was demonstrated that PP1c interacted with pRb via a KLRF sequence motif at its regulatory subunit binding site and PP1c was positioned in complex with pRb in a way that it could still exert its catalytic activity on phosphorylated residues [21]. In addition, binding of PP1c to pRb had another important consequence; it blocked interaction of pRb with CDKs; therefore, besides forming an active phosphatase-pRb complex, it might also contribute to the decreased phosphorylation level of pRb through keeping the kinase away from the substrate. The above results, together with previously published data [10,22–24] present compelling lines of evidence that PP1 is the major physiological phosphatase for pRb dephosphorylation. Nevertheless, many reports proved the involvement of PP2A in mediating the phosphorylation level of pRb [25,26] and other pocket proteins [27,28].

In this work, we have attempted to identify how PP1 and/or PP2A may influence the chemosensitivity of leukemic cells against chemotherapeutic drug treatments using cell-permeable phosphatase inhibitory toxins selective for PP2A or PP1. Our present data suggest that PP2A may regulate the phosphorylation level of Erk1/2, Akt and pRb, and it had an influence on caspase-3 activity, too. In contrast, PP1 appears to be involved in the direct dephosphorylation of pRb. It is demonstrated that a 25 kDa kinase-enhanced phosphatase inhibitor (KEPI)-like protein, with known inhibitory potency on both PP1c and MP, may also participate in the inhibition of PP1 in THP-1 cells, thereby increasing the phosphorylation level of pRb. Our results support the conclusion that PP2A influences pRb dephosphorylation indirectly, via regulating the phosphorylation state of PP1c inhibitory or regulatory proteins (KEPI, MYPT1). These data draw attention to the importance of the expression and phosphorylation of PP1 inhibitory proteins in malignant cells and on their role in controlling the phosphorylation state of key proteins in the regulation of cell survival.

2. Materials and methods

2.1. Cell cultures

THP-1 human monocytic leukemia cells, KG-1 human myeloid leukemia cells, MCF-7 breast cancer cells and HeLa human cervical cancer cells were purchased from the European Collection of Cell Cultures (ECACC) and cultured according to the supplier's recommendations at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air.

2.2. In vitro drug sensitivity assay

In vitro drug resistance assays were assessed using a 2-day cell cultures on microtiter plates. THP-1 or KG-1 cells were preincubated initially with 50 nM calyculin A (CLA, Santa Cruz Biotechnology) or 1 μM tautomycin (TM, Merck Millipore) and were washed with PBS after 1 h. Daunorubicin (DNR, Sigma-Aldrich) was dissolved in DMSO, and it was placed out in 50 nL volumes and four concentrations in triplicates on 384-well plates by using a Biomek robot. Each well was loaded with 30 μL cell suspension containing 1000 cells as the final concentrations of DNR were 0.15 μM, 0.61 μM, 1.84 μM and 7.38 μM. After 2 days of incubation, the live and dead cells were differentially stained using fluorescent VitalDye (Biomarker, Hungary). The precise number of living cells was determined using a custom built automated laser confocal fluorescent microscope (a modified Perkin-Elmer UltraView LCI) at the Karolinska Institute visualization core facility (KIVIF). The images were captured using the computer program QuantCapture 4.0, whereas the living cells were identified and individually counted using the program QuantCount 3.0. Both programs were developed at the KIVIF using OpenLab Automator programming environment (Improvision) [29]. Fifteen control wells that were used to determine the control cell survival contained cells with only culture medium and 50 nL solvent (DMSO) without drugs, 5 wells contained cells with culture medium alone. Comparing the two types of control wells, no toxic effect of DMSO could be seen. Mean cell survival was determined from the average of cell survival from the three identical wells.

2.3. Phosphatase activity assay

Prior to treatments, THP-1 cells were incubated in serum-free medium for 16 h. To investigate the influence of the inhibitors on phosphatase activity, cells were treated with 50 nM CLA and 1 μM TM for 1 h in serum-free media, and lysates were prepared for phosphatase assays as described previously [30]. Briefly, cells were washed with phosphate-buffered saline (PBS) followed by 0.1 M Tris–HCl (pH 7.6), 150 mM NaCl (TBS) containing 0.1 mM EDTA, and then collected in 100 μl ice-cold TBS containing 0.1 mM EDTA supplemented with 0.5% protease inhibitor cocktail and 50 mM 2-mercaptoethanol. Cells were frozen in liquid nitrogen and then thawed and sonicated, and the lysates were clarified by centrifugation at 16,000g for 10 min. The phosphatase activity of the supernatants (30-fold final dilution in the assays) was assayed with 1 μM ^{32}P-labeled 20 kDa light chain (^{32}P-MLC20) of turkey gizzard

Please cite this article as: D. Dedinszki, et al., Cell. Signal. (2014), http://dx.doi.org/10.1016/j.cellsig.2014.11.021

myosin [31] in the absence or presence of 2 μM His-inhibitor-2 (I-2, expressed and prepared as described in [21]) at 30 °C in 20 mM Tris–HCl (pH 7.4) and 0.1% 2-mercaptoethanol. The reaction was initiated by addition of the substrate. After a 10-min incubation, the reaction was terminated by the addition of 200 μl 10% TCA and 200 μl 6 mg/ml BSA. The precipitated proteins were collected by centrifugation and the released $^{32}P_i$ was determined from the supernatant (370 μl) in a scintillation counter.

2.4. Caspase-3 activity measurement

The activity of caspase-3 enzyme was measured in both treated and control cells using a fluorometric Caspase-3 Immunoassay/Activity Kit (Merck Millipore). After treatments, THP-1 cells (5×10^6) were harvested by centrifugation and washed with ice-cold PBS. Cells were lysed by adding 110 μL chilled lysis buffer (10 mM HEPES, 2 mM EDTA, 0.1% CHAPS, 1 mM PMSF, 10 μg/mL Pepstatin A, 10 μg/mL Aprotinin, 20 μg/mL Leupeptin). Lysates were centrifuged at 16,000*g* for 3 min at 4 °C. A 60-μM substrate in 2× reaction buffer (200 mM HEPES, 20% sucrose, 0.1% CHAPS) was added to the supernatant, and the samples were incubated for 60 min at 37 °C. The fluorescence from appropriately diluted samples was measured at 380 nm excitation and 460 nm emission.

2.5. Detection of normal, apoptotic and necrotic cells by differential staining and cell morphology as well as lactate dehydrogenase (LDH) assay

After treatments, the cells were washed two times with phosphate-buffered saline (PBS) and stained with 10 μM Hoechst 33342 (Sigma-Aldrich) in PBS for 15 min followed by staining with 5 μM propidium iodide (PI) for 5 min at 37 °C in the dark. For the analysis of cell viability and nuclear morphology, cells were seeded on poly-L-lysine-coated coverslips. Afterwards, coverslips were washed with PBS and mounted with ProLong Gold antifade reagent. Images were acquired with a Zeiss LSM 510 Meta confocal microscope (Carl Zeiss, Jena, Germany). The autofluorescent signal of DNR was excluded by detecting the PI emission in the wavelength range of 710 to 750 nm. Cells were classified as "viable" (Hoechst positive/PI negative, normal nuclear morphology), "apoptotic" (Hoechst positive/PI negative, chromatin condensation, fragmentation, blebbing) or "necrotic" (Hoechst positive/PI positive). At least 200 cells were examined in each preparation in three independent experiments.

The proportion of necrotic cells was also assessed by measuring the release of lactate dehydrogenase (LDH) from damaged cells using Cytoscan colorimetric assay (G-Biosciences) according to the manufacturer's instructions. Briefly, after treatments, cells were collected and centrifuged at 8000*g* for 1 min, and the supernatants (50 μl) were transferred into 96-well flat bottom plate. Substrate mix (50 μl) was added, and the plate was incubated (20 min) and then the absorbance of samples was measured at 490 nm. Culture medium without cells was used as background control. The maximum amount of releasable LDH activity was determined by disrupting the cells with lysis buffer. Cytotoxicity was expressed as a percentage of maximum LDH activity.

2.6. Cell treatment and Western blotting

THP-1 cells were serum starved for overnight before treatment with 50 nM CLA or 1 μM TM for 1 h. Then the cells were collected by centrifugation at 800*g*, the culture medium was discarded and the cells were incubated in fresh RMPI with or without 2 μg/mL DNR for 6 h. After the treatment, the THP-1 cells were collected by centrifugation (800*g*, 3 min) and washed with ice-cold phosphate-buffered saline (PBS) and lysed in 100 μL RIPA lysis buffer (50 mM Tris–HCl, 10 g/L Nonident P-40, 10 g/L Na-deoxycholate, 1 g/L Na-dodecyl sulfate (SDS), 0.15 M NaCl, 2 mM EDTA), containing 0.5% protease inhibitor mix (Roche) and 0.1 μM microcystine-LR.

MCF-7 cells were serum starved for overnight before treatment with 25 nM CLA for 1 h or 100 nM phorbol 12-myristate 13-acetate (PMA, Santa Cruz Biotechnology) for 30 min, harvested by a cell scraper and lysed in 100 μL RIPA lysis buffer supplemented with 0.5% protease inhibitor mix (Roche) and 0.1 μM microcystine-LR.

Cell lysis was accelerated by ultrasonic treatment; cell debris was removed by centrifugation (4 °C, 16000*g*, 10 min). Lysates were boiled at 100 °C for 10 min with 5× SDS sample buffer (320 mM Tris–Cl pH 6.8, 10% SDS, 50% glycerol, 25% β-mercaptoethanol, 0.01% bromophenol blue). Protein concentration of the lysates was measured by BCA protein assay (Thermo Scientific) at 540 nm in an ELISA Reader (Labsystem Multiscan MS). Equal amounts of cell lysates (50 μg) were loaded onto 10% or 15% SDS–polyacrylamide gel and subjected to SDS–PAGE then transferred to nitrocellulose membrane. Nonspecific binding sites were blocked with 5% nonfat dry milk in Tris-buffered saline (TBS) containing 0.5% Tween-20 (TBST). Membranes were probed for proteins of interest using primary antibodies to phospho-Thr38 of CPI-17 (1:500–1:1000) (Santa Cruz), phospho-Thr826 of retinoblastoma protein (1:1000) (Sigma-Aldrich), phospho-Thr202/Tyr204 of Erk1/2 (1:1000) (Cell Signaling Technology Inc.), phospho-Ser473 of Akt (1:1000) (Cell Signaling Technology Inc.), Flag-tag (1:3000) (Sigma-Aldrich), KEPI (1:1000) (Abcam), PP1c (1:1000) (Santa Cruz Biotechnology) and actin (1:2000) (Sigma-Aldrich). The membranes were washed two times with TBST and once with TBS for 10 min then incubated with horseradish peroxidase-conjugated rabbit or mouse secondary antibody (1:8000) (Sigma-Aldrich). The immunoreactive bands were detected by ECL reagents (Thermo Scientific) and imaged with FluorChem FC2 Imager (Alpha Innotech).

2.7. Transfection of siRNA

The panPP1 siRNA (Santa Cruz Biotechnology) and the appropriate DharmaFECT 2 transfection reagent (Thermo Scientific) were diluted in serum-free MEM media. siRNA was added to the transfection reagent and incubated for 10 min at room temperature. Then this mixture was added to HeLa cells in 100 nM final concentration of siRNA and incubated in suspension for 30 min. The cells were plated and the same volume of MEM media containing 20% FBS was added. Cells were incubated for 48 h before analysis.

2.8. Transfection of Flag-KEPI

MCF-7 cells were transfected with Flag-KEPI plasmid (GeneCopoeia) using Gene Juice transfection reagent (Merck Millipore) in a 6-well plate. The Flag-KEPI plasmid (2 μg/well) and the transfection reagent (6 μL/well) were diluted in 100 μL serum and antibiotic-free MEM medium in separate tubes and incubated for 5 min. Then the diluted plasmid was added to the diluted transfection reagent and the mixture was incubated for 10 min. Then these mixtures were added to the MCF-7 cells in serum-free media and incubated in suspension for 30 min. The cells were plated and the same volume of MEM media containing 20% FBS was added. Cells were incubated for 48 h.

2.9. Immunofluorescence microscopy

After 30 min incubation in suspension with the transfection mixture, cells were plated on coverslips 48 h before the immunofluorescent staining. Paraformaldehyde (3.7%) was used for fixation followed by three washes with PBS. Cells were permeabilized with 0.02% (v/v) Triton X-100 dissolved in PBS for 10 min at room temperature and washed three times with PBS. After incubation with 1% (w/v) bovine serum albumin (BSA) containing blocking solution for 1 h, cells were incubated with anti-Flag antibody diluted at 1:200 in 1% (w/v) BSA/PBS at room temperature for 2 h. After extensive washing with PBS (three times), cells were incubated with Alexa 488-conjugated chicken anti-rabbit antibody at a dilution of 1:200 and Texas Red Phalloidin (Molecular

Please cite this article as: D. Dedinszki, et al., Cell. Signal. (2014), http://dx.doi.org/10.1016/j.cellsig.2014.11.021

Probes) at a dilution of 1:1000 in 0.1% (w/v) BSA/PBS at room temperature for 1 h. Coverslips were washed three times with PBS and covered with mounting medium using SlowFade Antifade Kit (Life Technologies). Samples were imaged with Leica TCS SP8 Confocal Laser Scanning Microscope. Images were prepared using image-editing software (Photoshop; Adobe Systems).

2.10. Densitometry

For densitometry analysis of the Western blot images, ImageJ 1.46r software was used.

2.11. Statistical analysis

Experimental results were reported as a mean of at least three independent experiments. Statistical analysis was performed by *t* test, and a P value < 0.05 was considered statistically significant. Statistical analysis was carried out using GraphPad Prism software.

3. Results

3.1. The effect of CLA and TM on the survival of THP-1 and KG-1 leukemic cells upon DNR treatment

The functions of PP1 and PP2A in cellular regulation have been studied by the application of membrane permeable inhibitory toxins which exhibit selectivity toward these two types of phosphatase following cell permeation [30,32]. It was shown that 50 nM CLA specifically inhibited PP2A in Balb/c 3 T3 cells [32], while TM at 1 μM was believed to be selective for PP1 in several cell lines [30]. In the light of these results, we applied 50 nM CLA and 1 μM TM to THP-1 and KG-1 leukemia cells and studied their effects on the viability of these cells in the absence and presence of DNR, a potent chemotherapeutic drug used in treatments of leukemia. Both CLA and TM decreased viability to different extent for THP-1 (67–70%) and KG-1 cells (85–90%) as shown in Fig. 1A and B. DNR decreased cell viability in a concentration dependent manner, and at the highest DNR concentration survival was suppressed by close to 100% and 65% for THP-1 and KG-1 cells, respectively. When the cells were preincubated with CLA or TM before DNR treatment the cell death inducing efficacy of DNR was profoundly attenuated, suggesting that inhibition of either PP1 or PP2A results in decrease of the chemosensitivity of these leukemic cells toward DNR. Similar protective effects of CLA or TM against cell death inducing effect of other chemotherapeutic drugs (cytosine arabinoside, methotrexate, actinomycin-D) to THP-1 or KG-1 cells were also detected (data are not shown).

PP1 and PP2A are known to exert their regulatory influence via dephosphorylation of distinct substrates in the signaling pathways. The similar effects of CLA and TM on the cell survival of THP-1 and KG-1 cells in the presence of DNR might raise questions if these inhibitors exert their previously suggested type specificity in phosphatase inhibition [30,32] in THP-1 cells under our experimental conditions. Thus, after treatments of THP-1 cells with CLA or TM, the lysates were assayed for phosphatase activity in the absence or presence of 2 μM I-2 with ^{32}P-MLC20 substrate, which is dephosphorylated by both PP1 and PP2A (Fig. 2). I-2 specifically inhibits PP1; therefore, the I-2-sensitive phosphatase activity corresponds to PP1, while the I-2 insensitive fraction is due to PP2A. In untreated THP-1 cell lysate, I-2 reduced the phosphatase activity to 52 ± 0.95%, suggesting approximately equal activity ratio of PP1 and PP2A in these cells. CLA or TM treatment of THP-1 cells resulted in suppression of phosphatase activity of the lysates to 79 ± 5.9% and 61.7 ± 2.6%, respectively, when the assays were carried out in the absence of I-2. In the presence of I-2, CLA treatment decreased the I-2 insensitive phosphatase activity by similar extent (18.3%) to that observed in the absence of I-2 (21%), implying a PP2A-specific inhibitory influence of CLA in THP-1 cells. In contrast, TM treatment was essentially without effect on the I-2 insensitive phosphatase activity (only ~3% inhibition), suggesting that it specifically suppressed PP1 activity. The above results confirmed the previously claimed PP1 and PP2A type specificity of phosphatase inhibition [30,32] in THP-1 cells by TM and CLA as well. Similar specificities in the inhibition of PP1 and PP2A by TM and CLA were assessed in KG-1 and MCF-7 cells (data are not shown). It is to note also that the inhibitors (TM or CLA) in the applied concentrations resulted in specific, but only partial inhibition of PP1 and PP2A in these cells. Nevertheless, the extent of phosphatase inhibition they exerted could be expected to shift the kinase/phosphatase activity ratio significantly toward the preference of phosphorylation of several substrates.

3.2. Effect of DNR, CLA, TM and their combinations on caspase-3 activity and the type of cell death in THP-1 cells

The activation of caspase-3 is an indicator of apoptosis, and it was reported previously that DNR treatment increased caspase-3 activity and PP2A inhibition influenced caspase-3 activation [13]. Therefore, we examined if the PP2A and PP1 inhibitors themselves, or in the presence of DNR, affect caspase-3 activity differently in THP-1 cells. Fig. 3A illustrates that CLA and TM elevated caspase-3 activity significantly; however, the extent of activation was substantially higher in case of CLA (4.5-fold) compared to TM (1.5-fold). DNR enhanced caspase-3 activity profoundly (~8-fold), which was attenuated in the presence of CLA (~4-fold), but was not changed upon combination of TM with DNR.

It is generally assumed that the changes in caspase-3 activation correlates with the extent of apoptotic cell death. However, our data in Fig. 1 and Fig. 3A are controversial with regards of this assumption,

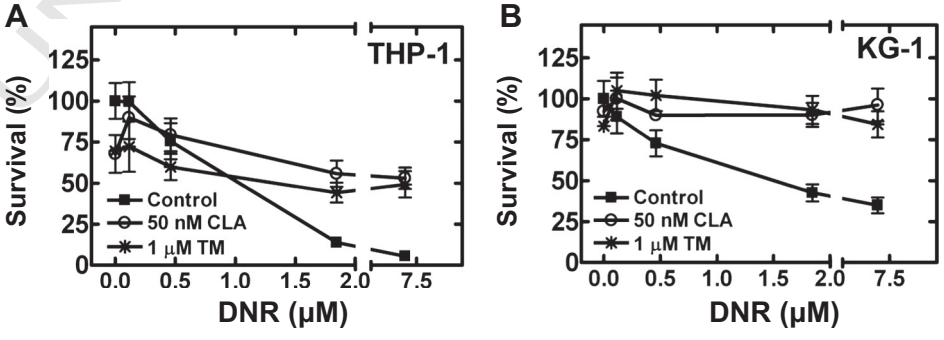

Fig. 1. Effect of CLA and TM on the survival of THP-1 and KG-1 cells in the absence or presence of DNR. Survival of THP-1 (A) and KG-1 (B) cells and the number of living and dead cells was determined using an automated laser confocal fluorescent microscope. THP-1 (A) and KG-1 (B) cells were pretreated with solvent, CLA (50 nM) or TM (1 μM) for 1 h, then incubated for 2 days with DNR at 4 different concentration (0.15 μM, 0.61 μM, 1.84 μM, 7.38 μM). Cell viability was determined using fluorescent VitalDye as described in the Materials and Methods section. Data represent the average of triplicates. Survival of the cells in the absence of effectors was taken as 100%.

Please cite this article as: D. Dedinszki, et al., Cell. Signal. (2014), http://dx.doi.org/10.1016/j.cellsig.2014.11.021

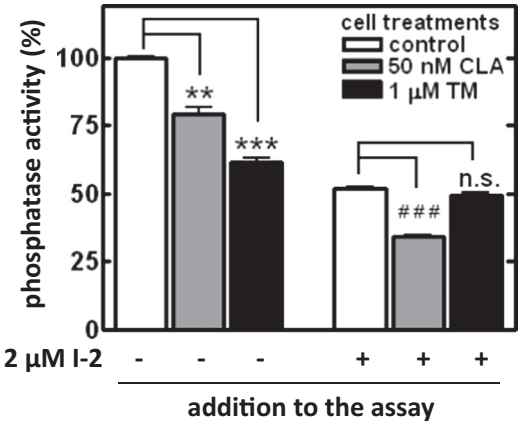

Fig. 2. Type specificity of phosphatase inhibition in CLA and TM treated THP-1 cells. THP-1 cells were incubated with 50 nM CLA or 1 μM TM for 1 h, then cells were washed and cell lysates were prepared for phosphatase assays as described in the Materials and Methods section. Phosphatase activity of the lysates was assayed in the absence or in the presence of 2 μM I-2 with 1 μM ^{32}P-MLC20 substrate. Phosphatase activity in the absence of the effectors was taken as 100%. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{###}p < 0.001$, n.s. not significant, Student *t* test.

especially in case of treatments involving TM, which did not influence the activation of caspase-3 by DNR, even though reduced profoundly DNR-induced cell death. These data raise questions concerning the ratio of the types of cell death (apoptosis and/or necrosis) upon the different treatments of THP-1 cells. Thus, cells treated with DNR or phosphatase inhibitors (CLA or TM) plus DNR were analyzed by Hoechst 33342 and propidium iodide (PI) staining as well as by assessing cell and nuclear morphology to determine the ratios of normal, apoptotic and necrotic cells (Fig. 3B). In control cells, approximately ~15% of apoptotic and necrotic cells were identified. DNR treatment increased the ratio of apoptotic cells to ~55%, while in the presence of CLA or TM apoptotic cells amounted to 30% or 32%, respectively. DNR and CLA increased the percentage of necrotic cells marginally, whereas TM was without effect. When preincubation of cells with CLA or TM was combined with DNR treatments the percentage of apoptotic cells was ~32-35% in accord with the previously observed attenuating effects of the phosphatase inhibitors on DNR-induced cell death. Lactate dehydrogenase (LDH) activity of differentially treated THP-1 cells was also assayed as a characteristic measure of the presence of necrotic cells (Fig. 3C). In accordance with the data of Fig. 3B control cells exhibited low level of LDH activity, which was moderately increased in the presence of DNR, CLA and CLA plus DNR, but was not affected by TM and TM plus DNR treatments. The above data suggest that upon the treatment of THP-1 cells with the chemotherapeutic agent DNR, the PP1 or PP2A inhibitor TM or CLA, or the combination of these drugs, the cells predominantly undergo apoptosis.

3.3. Effect of CLA and TM on the phosphorylation levels of Erk1/2, Akt, retinoblastoma protein and PP1 regulatory and inhibitory proteins in the absence and the presence of DNR

In order to study further the molecular background of the actions of protein phosphatase inhibitors, the phosphorylation states of several proteins important in cell survival were studied in the presence of CLA and TM. Fig. 4A depicts that Erk1/2 was not phosphorylated either in untreated or DNR-treated cells, whereas CLA induced the phosphorylation of Erk1/2 both in the absence and in the presence of DNR. Akt kinase showed about the same level of basal phosphorylation in the absence or presence of DNR, which was increased significantly upon CLA treatment. There was a basal phosphorylation at Thr826 residue of the pRb protein observed in control THP-1 cells, which was significantly reduced upon DNR treatment. In contrast, CLA treatment resulted

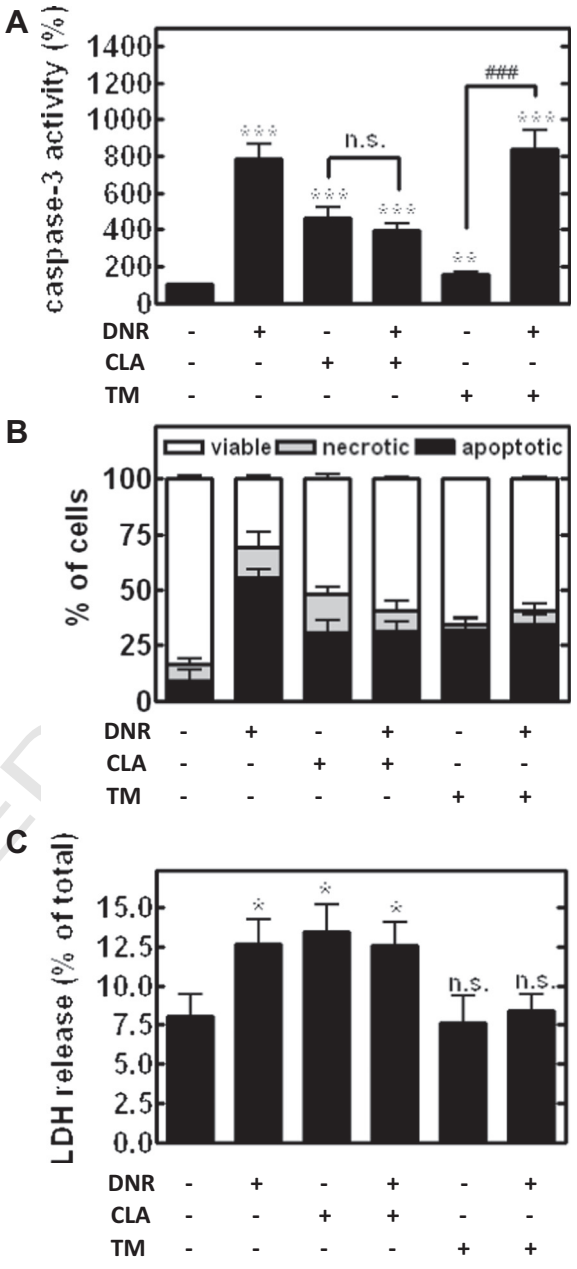

Fig. 3. Effect of DNR, CLA, TM and their combinations on caspase-3 activity and the proportion of apoptotic and necrotic cell death in THP-1 cells. THP-1 cells were untreated, treated with 50 nM CLA or 1 μM TM for 1 h and then with or without DNR, for 6 h. The caspase activity (A) in THP-1 cell lysates was determined using the caspase-3 activity assay as described in the Materials and Methods section. Caspase activity in the absence of the effectors was taken as 100%. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{###}p < 0.001$, n.s. not significant, Student *t* test. The ratio of normal, apoptotic and necrotic cells (B) and LDH release (C) was determined after 12 h treatment of THP-1 cells with DNR, CLA, TM and their combinations as described in the Materials and Methods section. $^{*}p < 0.05$, n.s. not significant, Student *t* test.

in a profound rise in the phosphorylation of Thr826 of pRb compared to control both in the absence and presence of DNR. These results suggest that the inhibition of PP2A in THP-1 cells increases the phosphorylation level and activation of Erk1/2 and Akt kinases as well as the phosphorylation of pRb, which are important survival factors and may contribute to the decreased cell death observed by the combined treatment with CLA and DNR compared to DNR alone. When TM was applied in similar experiments (Fig. 4B), different phosphorylation pattern of the studied proteins was observed. TM did not induce the phosphorylation of Erk1/2 or it did not influence the basal phosphorylation of Akt kinase.

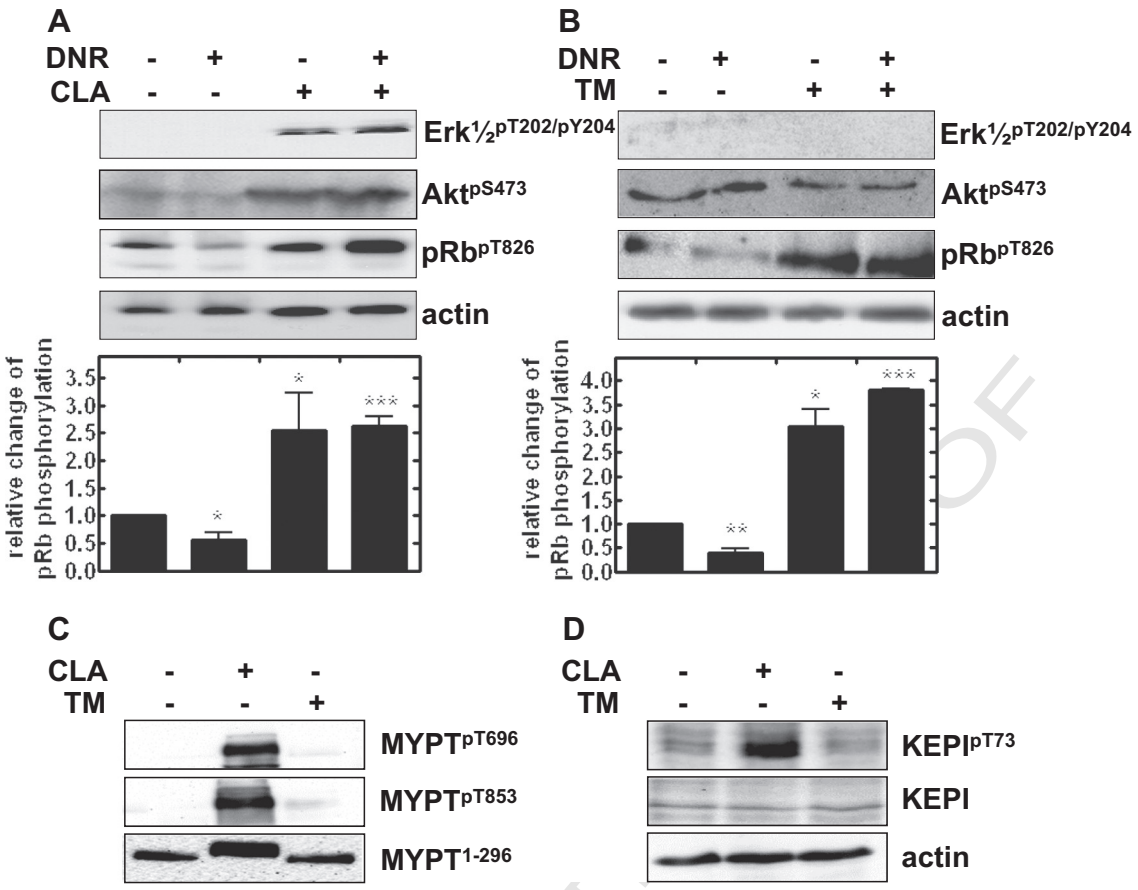

Fig. 4. Different effects of CLA and TM on protein phosphorylation in THP-1 cells. A and B: Phosphorylation of Erk, Akt and pRb in THP-1 cells. THP-1 cells were pretreated with 50 nM CLA (A) or 1 μM TM (B) and incubated in the absence or the presence of 2 μg/mL DNR for 6 h. Then the cells were lysed and subjected to Western blot analysis using Erk½$^{pT202/pY204}$, Akt^{pS473}, pRb^{pT826} antibodies. Histograms demonstrate densitometry analysis of pRb^{pT826} levels normalized to the corresponding densities of actin protein and the values represent the mean of three independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ Student t test. C: Phosphorylation of MYPT1 in THP-1 cells. THP-1 cells were untreated, treated with CLA or TM for 1 h. The membrane was probed with phosphorylation site-specific antibodies of MYPT (pT696 and pT853). Cross reactions with anti-MYPT$^{1-296}$ are shown as a loading control. D: Phosphorylation of a KEPI-like protein in THP-1 cells. Cells were untreated, treated with CLA or TM for 1 h. The membrane was probed with anti-CPI-17^{pThr38} antibody, which recognizes KEPI^{pT73} too, as well as anti-KEPI and anti-actin antibodies.

However, it increased the phosphorylation level of Thr826 in pRb similarly to that of CLA. These data are consistent with the conclusion that inhibition of PP1 by TM results in increased phosphorylation of pRb only. Since CLA and TM both attenuate the death of THP-1 cells similarly upon DNR treatment, the increased phosphorylation of pRb caused by these inhibitors may be a common pathway in their action. Previous findings also indicate that the mediation of pRb phosphorylation level is an important factor not only in the regulation of cell cycle, but also in the initiation of drug-induced apoptosis [10,33] as well as developing chemoresistance of leukemic cells [34] against chemotherapeutic drugs. Thus, these data together with our present results may implicate the phosphorylation/dephosphorylation events of pRb as important determinants in the chemoresistance of leukemic cells developed in the presence of phosphatase inhibitors. Therefore, in our further experiments, we investigated the molecular background of the pRb dephosphorylation processes with special emphasis on the type of phosphatase and regulatory proteins involved.

In order to find physiologically relevant inhibition of protein phosphatases, the possible activation of regulatory/inhibitory proteins by phosphorylation was investigated in THP-1 cells. We have identified the MP as a possible enzyme that acted on phosphorylated pRb in THP-1 cells [13] and showed that phosphorylation of the PP1 inhibitory sites (Thr696 and Thr853) in MYPT1 was parallel with increased phosphorylation of pRb. We confirmed this observation with respect to the effect of CLA on the phosphorylation on MYPT1 (Fig. 4C) and tested TM for a possible similar effect. It is apparent, however, that TM did not induce the phosphorylation of either inhibitory phosphorylation site in MYPT1. The role of MP in pRb dephosphorylation raises the question if MP inhibitory proteins such as C-kinase activated phosphatase inhibitory protein of 17 kDa (CPI-17) [35] or kinase-enhanced phosphatase inhibitor (KEPI) [36] is present and become phosphorylated in THP-1 cells. Therefore, we subjected control and CLA or TM challenged cells to Western blots to identify phosphorylated CPI-17/KEPI-like proteins using an antibody specific for the phosphorylated Thr38 of CPI-17, the site required to be phosphorylated for phosphatase inhibitory potency. A protein band at 25 kDa (p25) was identified which was cross-reactive with both an anti-KEPI and anti-CPI-17^{pThr38} antibodies (Fig. 4D). There was a basal level of phosphorylation of p25 in control cells, which was significantly increased upon CLA treatment, but remained essentially unchanged when the cells were exposed to TM. These data suggest that PP1 regulatory and inhibitory proteins are expressed in THP-1 cells, and they are phosphorylated in a PP2A dependent manner which is accompanied with their increased PP1 inhibitory potencies.

3.4. Silencing PP1c increases pRb phosphorylation in HeLa cells

The above results implicate PP1 and phosphorylatable PP1 inhibitory proteins in the dephosphorylation of pRb. To gain further support of this finding we attempted to silence PP1c and determine its influence on pRb phosphorylation. We have not succeeded to transfect THP-1 cells in order to silence PP1c with small interfering RNA (siRNA) or with

constructs to overexpress KEPI inhibitory protein, to obtain transfected cells in necessary ratios to reliably study their effects. Thus, we applied panPP1c siRNA to HeLa cells to model the effect of PP1 silencing on pRb phosphorylation. We successfully silenced PP1c in these cells to ~25% of control and it was apparent that the decreased expression of PP1c resulted in a rise in pRb phosphorylation at Thr826 (Fig. 5) confirming the essential role of PP1 in pRb dephosphorylation.

3.5. Expression and phosphorylation of Flag-KEPI in MCF-7 cells increases pRb phosphorylation

To elucidate the role of phosphatase inhibitory proteins in the mediation of pRb phosphorylation MCF-7 cells were transfected with Flag-KEPI construct to overexpress this protein. MCF-7 cells serve as good models for these experiments since it has been established previously that they do not express KEPI [37]. Fluorescence microscopy of MCF-7 cells with anti-Flag antibody indicated an average of 50% transfection ratio (data are not shown). As KEPI is a protein kinase C (PKC) potentiated protein we also probed the effect of the PKC activator phorbol myristate acetate (PMA) on the localization and phosphorylation of the expressed Flag-KEPI (Fig. 6). Confocal images of the cells indicated that Flag-KEPI mainly resided in the cytoplasm in untreated cells, but PMA challenge induced shuttling of Flag-KEPI to the nucleus (Fig. 6A). In addition, PMA induced phosphorylation of Flag-KEPI at the inhibitory phosphosite (Thr73) as judged by the antibody specific for both phosphorylated CPI-17^{pThr38} and KEPI^{pThr73} as reported earlier [38]. Moreover, the increased Flag-KEPI phosphorylation was also accompanied with a rise in the phosphorylation of Thr826 in pRb in MCF-7 cells (Fig. 6B).

Next, we examined the effect of CLA on the phosphorylation level of Flag-KEPI expressed in MCF-7 cells. CLA increased the inhibitory phosphorylation of Flag-KEPI to a significant extent (Fig. 7A). It was also of interest how the phosphorylation of pRb^{pThr826} was influenced in non-transfected or Flag-KEPI transfected MCF-7 cells upon CLA treatment. Fig. 7B shows that in non-transfected MCF-7 cells there was a slight increase in pRb^{pThr826} upon CLA treatment. In contrast, in Flag-KEPI transfected MCF-7 cells the level of pRb^{pThr826} was relatively higher than in non-transfected cells, presumably due to a slight, basal phosphorylation level of Flag-KEPI (see Fig. 7A) even in the absence of CLA. CLA treatment resulted in a profound increase in the level of pRb^{pThr826} suggesting that the KEPI and its phosphorylation may be an important determinant in the regulation of the phosphorylation level of pRb.

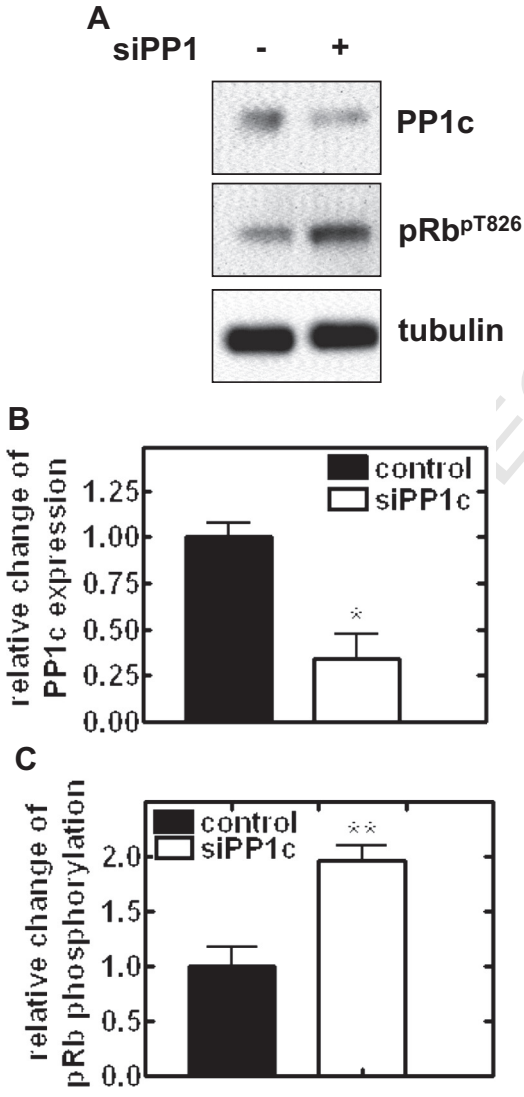

Fig. 5. Phosphorylation of pRb in response to PP1 gene silencing. PP1c was depleted in HeLa cells using siRNA-mediated gene silencing. Change of PP1c expression and the phosphorylation level of pRb were detected by Western blot analysis in control (incubated with transfection reagent, (−siRNA)) and siRNA transfected (+siRNA) cells (A). Data were quantified by densitometry and expressed as the PP1c/tubulin ratio (B) or as the pRb^{pT826}/tubulin ratio (C). *$p < 0.05$, **$p < 0.01$, Student t test.

4. Discussion

Our present data indicate that PP1 and PP2A play important roles in the regulation of drug-induced death of leukemic cells and they are also involved in controlling chemosensitivity against distinct drugs. It has been established that drug-induced apoptosis of malignant cells is accompanied with the activation of PP2A and PP1 [9,10]. In accordance with these observations our data show that DNR-induced cell death increases phosphatase activity in THP-1 leukemic cells as pRb phosphorylation was significantly reduced upon DNR treatment (see Figs. 4A and B). It is, therefore, not surprising that inhibition of PP1 by TM, or PP2A by CLA results in increased survival of THP-1 or KG-1 myeloid leukemia cells upon DNR treatment compared to that of DNR alone (Fig. 1). Assuming, however, that PP1 and PP2A may be involved in different signaling pathways with distinct substrate specificities it is intriguing that inhibition of either PP1 or PP2A leads to the same extent of increased survival of cells in the presence of DNR. For instances, our present results imply that PP2A- and PP1-specific inhibition have distinct effect on caspase-3 activation by DNR; therefore, they might act via different pathways in attenuating the extent of cell death upon DNR treatment. However, these data are also in conflict with the general view that the level of caspase-3 activation reflects the extent of apoptotic cell death during drug treatments. We have found here that during treatment of THP-1 cells by TM in combination with DNR the results obtained deviate considerably from this assumed correlation between caspase-3 activation and apoptotic cell death: TM did not influence caspase-3 activation by DNR; nevertheless it significantly decreased apoptotic cell death in the presence of DNR. The reasons for this discrepancy are not known, however, it is hypothesized that phosphatase inhibitors might influence the apoptotic pathways in a way to render them independent of caspase-3 activation. In support of this hypothesis it was previously reported [39] that okadaic acid (OA), another PP2A and PP1 inhibitor, induced apoptotic cell death of neutrophils and parallel increased caspase-3 activation, but even though apoptosis took place in a caspase-3 independent manner. Other possible alternatives are that phosphatase inhibitors can induce phosphorylation of caspase substrates which renders these proteins resistant to cleavage by this protease. Consistent with this assumption protection of pRb by its increased phosphorylation against caspase-3 cleavage has been previously reported [10,13].

Protein phosphorylation may influence the actual activity of a number of pro- and anti-apoptotic factors implicated in cell survival which could be the targets in the actions of PP2A and PP1. We studied changes

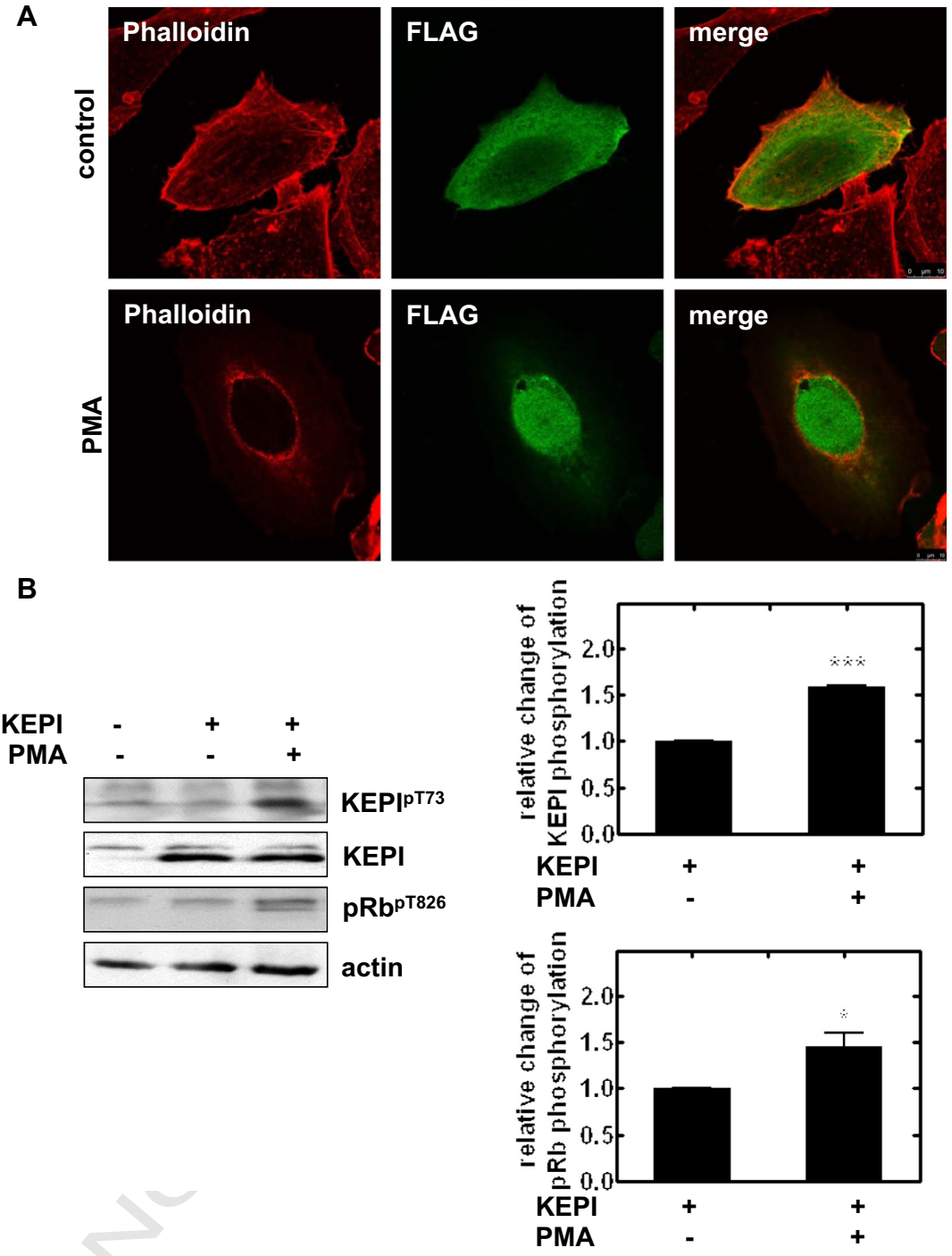

Fig. 6. The effect of PKC activation by PMA on the localization and phosphorylation of KEPI in Flag-KEPI transfected MCF-7 cells. (A) Localization of Flag-KEPI in MCF-7 cells. MCF-7 over-expressing Flag-KEPI were treated with 100 nM PMA for 30 min. The cells were fixed, stained with TexasRed–phalloidin (red) for actin and anti-Flag antibody for Flag-KEPI followed by indirect labeling with goat anti-rabbit IgG antibody conjugated with Alexa Fluor 488 (green), and then examined by confocal microscopy. (B) Phosphorylation of KEPI and pRb in Flag-KEPI transfected cells. MCF-7 cells were transfected with Flag-KEPI plasmid and then untreated or treated with PMA in a 100 nM final concentration for 30 min. Cells were lysed and the samples were subjected to Western blot analysis using antibodies against KEPI^{pT73}, KEPI, pRb^{pT826} and actin. Data were quantified by densitometry and expressed as the KEPI^{pT73}/KEPI ratio or as the pRb^{pT826}/actin ratio. *$p < 0.05$, ***$p < 0.001$, Student t test.

in the phosphorylation states of several such proteins and established that PP2A inhibition by CLA increased the phosphorylation level of p42/44Erk1/2 and Akt kinases which are known to be activated by phosphorylation, and PP2A has been implicated in their dephosphorylation processes [40]. In addition, PP2A inhibition increased the phosphorylation level of pRb and decreased DNR-induced caspase-3 activation. The anti-apoptotic effects of Erk1/2 and Akt activation [4–6] as well as the increased pRb phosphorylation and decreased caspase-3 activation [10] are well established, therefore, it is conceivable to assume that these PP2A regulated events could contribute to the increased cell survival. Nevertheless, the contribution of these processes to cell survival in our studied system is at question since PP1 inhibition by TM resulted in increased pRb phosphorylation only, even though the extent of increased survival was the same as in case of PP2A inhibition by CLA. The above data point to the pRb phosphorylation as a possible common thread in the regulatory actions of both PP1

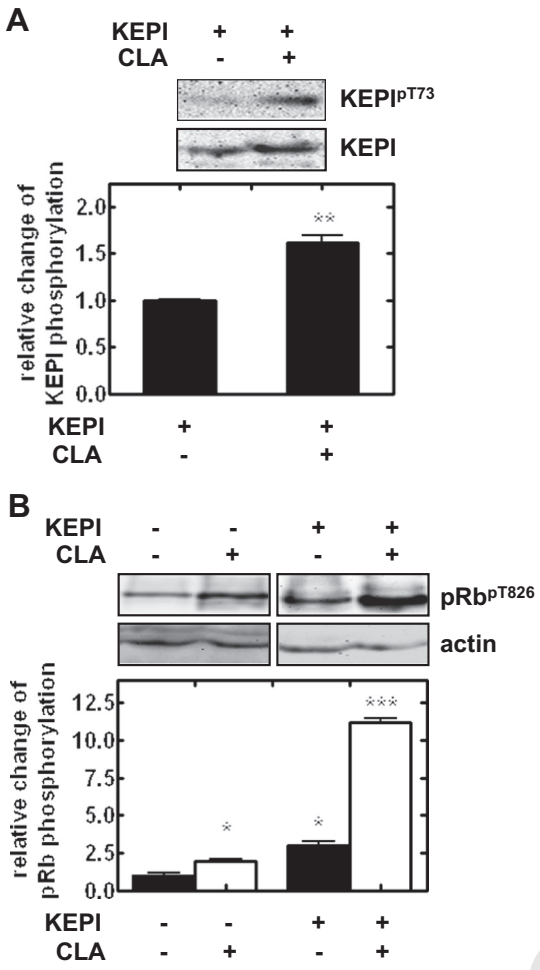

Fig. 7. Effect of CLA on the phosphorylation of KEPI and pRb in Flag-KEPI transfected MCF-7 cells. (A) MCF-7 cells were transfected with Flag-KEPI plasmid and treated with CLA (25 nM) for 1 h and KEPI^{pT73} protein was visualized by Western blot analysis. In the lower part, data were quantified by densitometry and expressed as the KEPI^{pT73}/KEPI ratio. $^{**}p < 0.01$, Student *t* test. (B) Non-transfected and Flag-KEPI transfected cells were untreated or treated with CLA for 1 h and the relative change of pRb phosphorylation was detected by Western blot. The histogram demonstrates densitometry analysis of pRb^{pT826} levels normalized to the corresponding densities of actin protein. $^{*}p < 0.05$, $^{***}p < 0.001$, Student *t* test.

and PP2A. Consistent with the importance of pRb in leukemic cell responses to drugs it was previously reported [41] that low pRb expression or hyperphosphorylated pRb in patients of acute myeloid leukemia was accompanied with increased leukemic cell proliferation and drug resistance with poor prognosis of survival. Furthermore, the amount and phosphorylation state of pRb appeared to be an important factor in the commitment of several leukemic cell lines to drug-induced apoptosis [42]. Chemotherapeutic drugs induce hypophosphorylation of pRb followed by its proteolytic cleavage due to phosphatase activation accompanying with the arrest of leukemic cells in G1 phase and initiation of apoptosis [10,33,34]. However, all of these events are effectively counteracted by inhibition of PP1 and PP2A by cell-permeable toxins indicating the importance of the elevated phosphorylation state of pRb in the survival and chemoresistance of leukemic cells. Implication of pRb (together with E2F1) in the transcriptional activation of proapoptotic genes has also been described [19] and phosphorylation of certain pRb sites appeared to be permissive for this pRb function. However, the influence of key phosphorylated residues (e. g. Thr821, Thr826) with these regards still remains to be elucidated.

Accumulating lines of evidence suggest that PP1 is the major pRb phosphatase in cells [10,22–24], however, the mechanisms via PP1 dephosphorylates pRb are still not clearly established and alternative routes might exist [13,21,43]. PP1c binds tightly to the C-terminal region of pRb to a PP1c-binding motif-like sequence and forms an active complex functional with respect to dephosphorylation of phosphorylated residues in pRb [21]. However, a competition between PP1c regulatory subunits and pRb for PP1c-binding is assumed [13] and the interaction of PP1c with distinct regulatory proteins may have variable effects on pRb dephosphorylation. Association of PP1c with PNUTS decreases PP1c activity on pRb [43]. In contrast, interaction of PP1c with MYPT1 increases the dephosphorylation rate of pRb suggesting that MP holoenzyme may also function as a pRb phosphatase [13]. Our data indicate that inhibitory phosphorylation of MYPT1 and a 25 kDa KEPI-like (CPI-17 family) PP1 inhibitory protein induced by CLA treatment parallels with the increased phosphorylation of pRb at Thr826 (see Figs. 4C and D). This influence of CLA may due to the inhibition of PP2A in accordance with previous data designating PP2A as one of the possible MYPT1 [44–46] and CPI-17 phosphatase type [44]. TM is without effect on the inhibitory phosphorylation of MYPT1 or the KEPI-like protein and this may indicate that (i) the auto-dephosphorylation of MYPT1^{pThr696} and/or MYPT1^{pThr853} in the PP1c-MYPT1 complex by PP1c, which has been suggested recently [47], is not inhibited by TM in THP-1 cells; (ii) the KEPI-like protein is not dephosphorylated by PP1. Our data support the conclusions that PP1 plays an essential role in pRb dephosphorylation and in this process phosphorylation of PP1 inhibitory proteins is an important regulatory factor. Secondly, PP2A may affect pRb dephosphorylation via an indirect way by controlling the PP1c activity in MP by dephosphorylation of MYPT1 and PP1c inhibitory proteins. The novel aspects of our study are that a KEPI-like inhibitory protein is present in THP-1 cells and it may be implicated in the regulation of pRb dephosphorylation. KEPI was shown to inhibit potently both PP1c and MP [38], the phosphatase forms considered to dephosphorylate pRb [13,21], therefore its physiological phosphorylation and increase of its inhibitory potency could have a major impact on the phosphorylation of pRb. It is to note that the basal phosphorylation of pRb appears to be relatively high in THP-1 cells and this might be due to a significant basal phosphorylation of KEPI-like protein (see Fig. 4D), but not MYPT1 (see Fig. 4C), consistent with a partial inhibition of pRb dephosphorylation in leukemic cells coupled with higher proliferation rate.

The changes in pRb phosphorylation was followed by the determination of the phosphorylation level of Thr826 which is considered to reflect major alteration in the interaction of pRb with physiological binding partners [20]. It should be noted, however, that phospho-Thr826 was also shown to be dephosphorylated by a specific trimeric form of PP2A activated by redox imbalance in cells and this holoenzyme includes a Ca^{2+}-sensitive 70 kDa regulatory B subunit [26]. Inhibition of this PP2A form by CLA might also contribute to the increased pRb phosphorylation. It is not known, however, if this PP2A holoenzyme is present in THP-1 cells and the conditions are appropriate for its activation, since the latter requires oxidative stress stimuli. Nevertheless, the role of PP1 in the dephosphorylation of pRb^{pThr826} may not be questioned since our present results substantiate that silencing of PP1c in HeLa cells (see Fig. 5) increases significantly the pRb^{pThr826} level implying an essential contribution of PP1 to this regulatory event.

We have provided further proofs in this study for the involvement of KEPI in the regulation of the phosphorylation level of pRb by investigating the effect of transfection of a Flag-KEPI construct to MCF-7 cells. We chose MCF-7 cells as a model system for these experiments as it was previously reported [37] that this cell line does not express KEPI. Thus, expression of KEPI in MCF-7 cells seemed to be reasonable as its effect could be studied without the influence of any endogenous protein. Our data imply that expression of Flag-KEPI in MCF-7 cells followed by challenging the cells with PMA for the activation of PKC, results in phosphorylation of Flag-KEPI at the PP1c inhibitory site and this event is coupled with increase in the level of pRb^{pThr826} (see Fig. 6B). Furthermore, PMA treatment induced translocation of Flag-KEPI from the cytoplasm to the nucleus, implicating PKC-dependent phosphorylation not

only in the activation of the inhibitor but also in the regulation of KEPI localization, too (Fig. 6A). This latter result is also supportive for the role of KEPI in the regulation of PP1 in the dephosphorylation of nuclear substrates, and pRb was also shown to reside predominantly in the nucleus of malignant cells [13]. Moreover, the role of inhibition of PP2A by CLA in increasing the level of phosphorylation at the phosphatase inhibitory site in Flag-KEPI is confirmed in MCF-7 cells (Fig. 7A). It is apparent that the expression of KEPI and inducing its phosphorylation by CLA result in a marked increase in the phosphorylation level of $pRb^{pThr826}$ in KEPI transfected MCF-7 cells compared to that of non-transfected cells (Fig. 7B). Our data are consistent with previous findings [48], indicating that overexpression of a phosphorylation-dependent human inhibitor-5 of protein phosphatase-1 in SW480 cells promotes growth and accelerates G1/S transition, which is accompanied with increased pRb phosphorylation. Moreover, CPI-17, originally identified as a PKC-dependent PP1c and MP inhibitor, is considered as a tumor promoter since it is overexpressed in some cancer cells and inhibits dephosphorylation of merlin by MP [49]. The above data collectively suggest that expression and phosphorylation of PP1 inhibitory proteins may be important determinant in the regulation of the phosphorylation level of proteins in malignant cells.

Acknowledgment

This work was supported by the following grants: OTKA K109249 (to FE) and PD111715 from the Hungarian Scientific Research Fund; TÁMOP-4.2.2/B-10/-1-2010-0024, TÁMOP-4.2.2.A-11/1/KONV-2012-0025 and TÁMOP 4.2.4. A/2-11-1-2012-0001 'National Excellence Program' supported by the European Union and the State of Hungary, co-financed by the European Social Fund. The authors are indebted to Mrs. Ágnes Németh for excellent technical assistance.

References

[1] R.G. Wickremasinghe, A.V. Hoffbrand, Blood 93 (1999) 3587–3600.
[2] X. Cheng, R.L. Bennett, X. Liu, M. Byrne, W. Stratford May, Blood Cancer J. 3 (2013) e144.
[3] S. Grethe, M.I. Porn-Ares, Cell. Signal. 18 (2006) 531–540.
[4] P. Dent, S. Grant, Clin. Cancer Res. 7 (2001) 775–783.
[5] L.S. Steelman, R.A. Franklin, S.L. Abrams, W. Chappell, C.R. Kempf, J. Basecke, F. Stivala, M. Donia, P. Fagone, F. Nicoletti, M. Libra, P. Ruvolo, V. Ruvolo, C. Evangelisti, A.M. Martelli, J.A. McCubrey, Leukemia 25 (2011) 1080–1094.
[6] A.M. Martelli, C. Evangelisti, W. Chappell, S.L. Abrams, J. Basecke, F. Stivala, M. Donia, P. Fagone, F. Nicoletti, M. Libra, V. Ruvolo, P. Ruvolo, C.R. Kempf, L.S. Steelman, J.A. McCubrey, Leukemia 25 (2011) 1064–1079.
[7] A. Garcia, X. Cayla, J. Guergnon, F. Dessauge, V. Hospital, M.P. Rebollo, A. Fleischer, A. Rebollo, Biochimie 85 (2003) 721–726.
[8] A.M. Smith, K.G. Roberts, N.M. Verrills, in: D.S. Koschmieder, Dr SteffenKoschmieder (Eds.), Myeloid Leukemia—Basic Mechanisms of Leukemogenesis, InTech, 2011.
[9] P.P. Ruvolo, Leukemia 15 (2001) 1153–1160.
[10] R.H. Wang, C.W. Liu, V.I. Avramis, N. Berndt, Oncogene 20 (2001) 6111–6122.
[11] K. Chatfield, A. Eastman, Biochem. Biophys. Res. Commun. 323 (2004) 1313–1320.
[12] R.T. Boudreau, D.M. Conrad, D.W. Hoskin, Cell. Signal. 19 (2007) 139–151.
[13] A. Kiss, B. Lontay, B. Bécsi, L. Márkász, E. Oláh, P. Gergely, F. Erdődi, Cell. Signal. 20 (2008) 2059–2070.
[14] R.A. Siddiqui, L.J. Jenski, K. Neff, K. Harvey, R.J. Kovacs, W. Stillwell, Biochim. Biophys. Acta 1499 (2001) 265–275.
[15] A.S. Harmala-Brasken, A. Mikhailov, T.S. Soderstrom, A. Meinander, T.H. Holmstrom, Z. Damuni, J.E. Eriksson, Oncogene 22 (2003) 7677–7686.
[16] L. Khidr, P.L. Chen, Oncogene 25 (2006) 5210–5219.
[17] C. Giacinti, A. Giordano, Oncogene 25 (2006) 5220–5227.
[18] F.A. Dick, S.M. Rubin, Nat. Rev. Mol. Cell Biol. 14 (2013) 297–306.
[19] A. Ianari, T. Natale, E. Calo, E. Ferretti, E. Alesse, I. Screpanti, K. Haigis, A. Gulino, J.A. Lees, Cancer Cell 15 (2009) 184–194.
[20] V. Kolupaeva, V. Janssens, FEBS J. 280 (2013) 627–643.
[21] A. Hirschi, M. Cecchini, R.C. Steinhardt, M.R. Schamber, F.A. Dick, S.M. Rubin, Nat. Struct. Mol. Biol. 17 (2010) 1051–1057.
[22] D.A. Nelson, N.A. Krucher, J.W. Ludlow, J. Biol. Chem. 272 (1997) 4528–4535.
[23] S. Tamrakar, S. Mittnacht, J.W. Ludlow, Oncogene 18 (1999) 7803–7809.
[24] E. Rubin, S. Mittnacht, E. Villa-Moruzzi, J.W. Ludlow, Oncogene 20 (2001) 3776–3785.
[25] J. Garriga, A.L. Jayaraman, A. Limon, G. Jayadeva, E. Sotillo, M. Truongcao, A. Patsialou, B.E. Wadzinski, X. Grana, Cell Cycle 3 (2004) 1320–1330.
[26] A. Magenta, P. Fasanaro, S. Romani, V. Di Stefano, M.C. Capogrossi, F. Martelli, Mol. Cell. Biol. 28 (2008) 873–882.
[27] L. Cicchillitti, P. Fasanaro, P. Biglioli, M.C. Capogrossi, F. Martelli, J. Biol. Chem. 278 (2003) 19509–19517.
[28] K.J. Soprano, E. Purev, S. Vuocolo, D.R. Soprano, Oncogene 25 (2006) 5315–5325.
[29] L. Márkász, G. Stuber, E. Flaberg, A.G. Jernberg, S. Eksborg, É. Oláh, H. Skribek, L. Székely, BMC Cancer 6 (2006) 265.
[30] B. Favre, P. Turowski, B.A. Hemmings, J. Biol. Chem. 272 (1997) 13856–13863.
[31] F. Erdodi, B. Toth, K. Hirano, M. Hirano, D.J. Hartshorne, P. Gergely, Am. J. Physiol. 269 (1995) C1176–C1184.
[32] Y. Yan, M.C. Mumby, J. Biol. Chem. 274 (1999) 31917–31924.
[33] Q.P. Dou, B. An, P.L. Will, Proc. Natl. Acad. Sci. U. S. A. 92 (1995) 9019–9023.
[34] Q.P. Dou, V.W. Lui, Cancer Res. 55 (1995) 5222–5225.
[35] M. Eto, S. Senba, F. Morita, M. Yazawa, FEBS Lett. 410 (1997) 356–360.
[36] Q.R. Liu, P.W. Zhang, Q. Zhen, D. Walther, X.B. Wang, G.R. Uhl, J. Biol. Chem. 277 (2002) 13312–13320.
[37] K. Wenzel, K. Daskalow, F. Herse, S. Seitz, U. Zacharias, J.A. Schenk, H. Schulz, N. Hubner, B. Micheel, P.M. Schlag, K.J. Osterziel, C. Ozcelik, S. Scherneck, B. Jandrig, Biol. Chem. 388 (2007) 489–495.
[38] F. Erdődi, E. Kiss, M.P. Walsh, B. Stefansson, J.T. Deng, M. Eto, D.L. Brautigan, D.J. Hartshorne, Biochem. Biophys. Res. Commun. 306 (2003) 382–387.
[39] H.Y. Park, M.G. Song, J.S. Lee, J.W. Kim, J.O. Jin, J.I. Park, Y.C. Chang, J.Y. Kwak, J. Cell. Physiol. 212 (2007) 450–462.
[40] M.J. Van Kanegan, D.G. Adams, B.E. Wadzinski, S. Strack, J. Biol. Chem. 280 (2005) 36029–36036.
[41] S.M. Kornblau, M. Andreeff, S.X. Hu, H.J. Xu, S. Patel, A. Theriault, C. Koller, H. Kantarjian, E. Estey, A.B. Deisseroth, W.F. Benedict, Clin. Cancer Res. 4 (1998) 1955–1963.
[42] B. An, Q.P. Dou, Cancer Res. 56 (1996) 438–442.
[43] G. De Leon, T.C. Sherry, N.A. Krucher, Cancer Biol. Ther. 7 (2008) 833–841.
[44] N. Takizawa, N. Niiro, M. Ikebe, FEBS Lett. 515 (2002) 127–132.
[45] B. Lontay, A. Kiss, P. Gergely, D.J. Hartshorne, F. Erdődi, Cell. Signal. 17 (2005) 1265–1275.
[46] B. Kolozsvári, É. Bakó, B. Bécsi, A. Kiss, Á. Czikora, A. Tóth, G. Vámosi, P. Gergely, F. Erdődi, Cardiovasc. Res. 96 (2012) 494–503.
[47] M. Khasnis, A. Nakatomi, K. Gumpper, M. Eto, Biochemistry 53 (2014) 2701–2709.
[48] X. Wang, B. Liu, N. Li, H. Li, J. Qiu, Y. Zhang, X. Cao, J. Biol. Chem. 283 (2008) 12076–12084.
[49] H. Jin, T. Sperka, P. Herrlich, H. Morrison, Nature 442 (2006) 576–579.

Please cite this article as: D. Dedinszki, et al., Cell. Signal. (2014), http://dx.doi.org/10.1016/j.cellsig.2014.11.021