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Caffeine activates tumor suppressor PTEN in sarcoma cells

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Abstract

The tumor suppressor phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is a negative regulator of the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway. Akt activation exerts a strong anti-apoptotic effect and inhibits key pro-apoptotic proteins. We investigated the effect of caffeine in the prevention of tumor cell proliferation and induction of cell death. We found that caffeine induced increased intracellular cAMP levels, PTEN activation, and Akt inactivation, which together prevented proliferation of human osteosarcoma cells (MG63) and fibrosarcoma cells (HT1080). PTEN-knockdown by siRNA reduced the effects of caffeine on Akt inactivation in osteosarcoma cells. These results indicate that the tumor suppressor PTEN signaling pathway contributes to the

growth-inhibitory effect of caffeine on sarcoma cells. Our data suggests that caffeine and other drugs that act on this pathway could have promising therapeutic effects in the treatment of sarcoma patients.

Introduction

Caffeine, a methylxanthine derivative, increases cAMP by inhibiting phosphodiesterase (PDE) activity, and is well-known to have diuretic and central-nervous system stimulatory effects. On the other hand, caffeine is an inhibitor of ataxia telangiectasia mutated (ATM) and ATM-Rad3-related (ATR) kinases, which are master regulators of DNA damage-induced cell cycle checkpoints (1,2). Therefore, caffeine has both anti-cancer or anti-carcinogenic effects and the ability to enhance the cytocidal activity of anticancer drugs by inhibiting DNA repair pathways (3-5). Based on these studies, we developed caffeine-assisted chemotherapy for bone and soft tissue sarcoma. While the five-year survival rates of patients with osteosarcomas are still only 60-70% (6-8), we previously reported that caffeine-assisted chemotherapy induced a complete response in more than 80% of patients with osteosarcoma (9), and an enhancement of the five-year survival rate to approximately 90% (10). These results indicate that caffeine is a beneficial agent for the treatment of malignant bone and soft tissue tumors such as osteosarcoma (11-14), but the molecular mechanism of caffeine in these diseases has not yet been fully elucidated.

The tumor suppressor gene *PTEN* (phosphatase and tensin homolog deleted in chromosome 10, 10q23.3) protein has been identified as a tumor suppressor in cancers of the prostate, breast, and endometrium (15-17). PTEN is a pivotal protein that regulates the balance between cell growth and death. PTEN and Phosphoinositide 3-kinase (PI3K) have

opposing effects on Akt regulation (18, 19). Akt in turn inhibits several downstream targets including the apoptosis-inducing protein BAD (20), p27 (21), and cyclin-dependent kinase (22), resulting in cell growth and survival.

We speculated that PTEN may be involved in the molecular mechanisms that are responsible for caffeine's antitumor effects. In this study, we found that caffeine activated PTEN by elevating intracellular cAMP levels, resulting in inhibition of Akt activity and induction of apoptosis.

Materials and Methods

Reagents and antibodies

RPMI-1640 medium with L-glutamine, phenol red, caffeine, and the PDE inhibitor, isobutylmethylxanthine (IBMX), were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan); fetal bovine serum (FBS) was from Sigma (St. Louis, MO, USA); and penicillin and streptomycin were from Gibco-Invitrogen (Carlsbad, CA, USA). Cisplatin was from Nippon Kayaku (Tokyo, Japan). The adenylate cyclase stimulator, forskolin, and vasodilator stimulated phosphoprotein (VASP) were obtained from Calbiochemical (La Jolla, CA). Antibodies with the following specificities were obtained from Cell Signaling Technology, Inc. (Beverly, MA): PTEN, phospho-specific PTEN (Ser380), Akt, and phospho-specific Akt (Ser473), β-actin, phospho-specific VASP, and cleaved caspase 3.

Cell culture

Human osteosarcoma cell line MG63 and human fibrosarcoma cell line HT1080 were obtained from the American Type Culture Collection (Rockville, MD). Both cell lines were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, penicillin and

streptomycin at 37°C in 5% CO_2.

Cell proliferation assay

Cell proliferation was analyzed with Cell Counting Kit 8 (DOJINDO, Japan). MG63 and HT1080 cells were seeded in 96 well plates at a density of 1×10^4 cells/well. After 24 h incubation, the cells were treated or not treated with 0.25-5 mM caffeine, or 1 mM IBMX, and/or 2 μg/ml cisplatin for 72 h. IBMX, a PDE inhibitor, was used in this assay to demonstrate that PDE-inhibition participates in the inhibitory effect of caffeine on cell proliferation. The cells were incubated with 10 μl WST-8 for 2 h. Absorbance of the colored formazan product produced by mitochondrial dehydrogenases in metabolically active cells was recorded at 450 nm as background. Cell proliferation was expressed as the percentage of absorbance obtained in the treated wells relative to that in the untreated control wells. The concentration of caffeine corresponding to 50% cell death indicates IC_{50}.

PTEN and Akt activity assays

To determine the effect of caffeine on PTEN and Akt activities, we investigated the levels of phospho-PTEN and phospho-Akt following various stimulations. Increases in phospho-PTEN/phospho-Akt levels indicated PTEN-inactivation and Akt-activation. MG63 cells were incubated in RPMI without serum for 24 h, and treated with caffeine (0, 1, 5 mM), IBMX (0.1, 1 mM) and/or forskolin (0.1 mM) for 30 min. Cells extracts were immunoblotted using anti-phospho-PTEN (Ser380), anti-PTEN, anti-phospho-Akt (Ser473), anti-Akt antibodies or anti-phospho-VASP. In this assay, phospho-VASP and forskolin were used to determine that the proliferation-inhibitory effect of caffeine depends on increases in cAMP levels.

Western blot analysis

Western blot analyses were performed as described previously (23, 24). All antibodies were used at a dilution of 1:1000.

Silencing of PTEN with small interfering RNA (siRNA).

Single-stranded human PTEN specific sense and antisense RNA oligonucleotides and control scrambled oligonucleotide were synthesized by *in vitro* transcription using the siTrio Full Set (# SHF27A-1888, B-Bridge International, Inc. USA) and annealed to generate a RNA duplex. The MG63 cells were transfected with small interfering RNA (100 μM) using Lipofectamine 2000 before experiments.

Statistical analysis.

Data are presented as means ± SEM from at least three independent experiments. Statistical analysis was performed by ANOVA followed by Dunnett's test.

Results

Caffeine inhibits proliferation of MG63 cells in a dose-dependent manner.

The proliferation of caffeine-treated MG63 osteosarcoma cells decreased in a dose-dependent fashion (IC_{50}= 2.70 mM) (Figure 1A).

Caffeine decreases the amount of phosphorylated PTEN and Akt in MG63 cells.

Caffeine treatment of MG63 cells also decreased the levels of phospho-PTEN (inactivated-PTEN) and phospho-Akt (activated-Akt) in a dose-dependent manner (Figure 1B, C), with the phosphorylated protein levels dropping by more than 50% while the overall protein amount remained constant. These results suggest that caffeine induces concentration-dependent activation of PTEN and inactivation of Akt.

PTEN depletion prevents caffeine-mediated Akt inhibition.

To obtain direct evidence that dephosphorylated PTEN is required for Akt inactivation by caffeine, we tested the effects of caffeine when PTEN protein was depleted by small interfering RNA. Akt phosphorylation was decreased by caffeine in control cells, but in PTEN knock-down cells caffeine-induced Akt inhibition was significantly reduced (Figure 1D). This result confirmed that Akt inactivation by caffeine is dependent on PTEN activation.

Caffeine and IBMX inhibit cell proliferation, phosphorylation of PTEN and Akt, and induce phosphorylation of VASP in MG63 cells.

Proliferation of MG63 cells was inhibited by caffeine and the PDE inhibitor IBMX (Figure 2A). In contrast, the level of phospho-VASP was increased by caffeine or IBMX (Figure 2B). VASP is a crucial factor in regulating actin dynamics, and the increased amount of phospho-VASP is an indication of the elevated intracellular cAMP concentrations (25). Since levels of phospho-PTEN and phospho-Akt were decreased by caffeine or IBMX in MG63 cells (Figure 2C, D), the increased cAMP levels that result from PDE inhibition might lead to PTEN activation, Akt inactivation, and subsequently inhibition of MG63 cell proliferation.

Forskolin supports the inhibition of Akt/PTEN phosphorylation by caffeine in MG63 cells.

To confirm that increased cAMP decreases the amount of phospho-PTEN and phospho-Akt, we tested the effects of forskolin, caffeine, and IBMX on MG63 cells. Caffeine and IBMX inhibit PDE, resulting in increased intracellular cAMP, while forskolin increases cAMP levels by stimulating adenylate cyclase. The level of phospho-VASP was slightly increased by a low dose of forskolin (Figure 3A), as well as low doses of caffeine or IBMX (data not shown). However, low doses of forskolin together with caffeine or IBMX strongly increased the amount of phospho-VASP, indicating that the combination of low doses of forskolin and

caffeine/IBMX synergistically increased intracellular cAMP (Figure 3A). These results suggest that forskolin leads to increased cAMP levels, which are immediately reduced because of the high PDE activity in MG63 cells. This possibility is supported by our results showing that treatment of MG63 cells with forskolin alone only slightly reduced the levels of phospho-Akt and phospho-PTEN (Figure 3B, C). The levels of phospho-PTEN and phospho-Akt were markedly decreased by combined treatment with forskolin and caffeine. Treatment with IBMX in combination with forskolin also significantly decreased the amounts of phospho-PTEN and phospho-Akt. Together, these results suggest that increased cAMP induces activation of PTEN and inactivation of Akt.

Caffeine decreases the level of phosphorylated PTEN and Akt in human fibrosarcoma HT1080 cells.

As with MG63 cells, the proliferation of HT1080 fibrosarcoma cells was reduced by caffeine in a dose-dependent manner (IC50=2.22 mM), and also by IBMX (Figure 4A). In addition, the level of phospho-VASP in HT1080 cells was also increased by caffeine and IBMX, and the amounts of phospho-PTEN and of phospho-Akt were decreased by caffeine and IBMX (Figure 4B). These results suggest that the caffeine-induced increase of cAMP may lead to PTEN activation, Akt inactivation, and inhibition of proliferation in HT1080 cells as well.

Caffeine enhances the antitumor effect of cisplatin on MG63 cells.

Proliferation of MG63 cells treated with 0.5 mM caffeine or 2 μg/ml cisplatin decreased by ~20% and 40%, respectively (Figure 5A), while the proliferation of cells treated with both cisplatin and caffeine was markedly decreased by more than 80%. The amount of cleaved caspase 3, an apoptosis marker, was increased by treatment with cisplatin, and markedly increased by treatment with both caffeine and cisplatin (Figure 5B). In contrast levels of

phospho-PTEN and phospho-Akt were significantly decreased by the treatment of cells with a combination of caffeine and cisplatin (Figure 5B). These results indicate that caffeine enhances the induction of apoptosis by cisplatin, confirming that our caffeine-assisted chemotherapy enhanced the treatment of osteosarcoma.

Discussion

Our results provide new insight into PTEN activation in the context of caffeine assisted sarcoma treatments (Figure 6). Intracellular cAMP also plays an important role in caffeine-regulated PTEN activation. PTEN, a lipid phosphatase specific for phosphatidylinositol (3, 4, 5)P_3 (PIP3), and PI3K have opposing effects on cellular PIP3 levels and consequently regulate cell proliferation and survival through various signaling molecules, especially Akt. Dephosphorylation of PIP3 by PTEN leads to inhibition of Akt activity. In this study, we showed that caffeine-induced increases in intracellular cAMP activate PTEN, in turn inhibiting Akt (Figure 1, 4) and subsequently inducing caspase-3 activation and apoptosis (Figure 5).

Osteosarcoma is the most common primary malignant bone tumor in children and young adults. During the last few decades, the prognosis of patients with osteosarcoma has been remarkably improved by a combination of surgical and chemotherapeutic treatments (6). At our institute, caffeine-assisted multi-agent chemotherapy improved the treatment success of malignant bone and soft tissue tumors such as osteosarcoma (10). While conventional multi-agent chemotherapies yield a local tumor response of approximately 63% and a five-year survival of 67.5% for non-metastatic osteosarcoma (6), the response rate of caffeine-assisted chemotherapy was over 80%, and the five-year survival rate was 90%

(five-year event-free rate, 76%) for primary non-metastatic osteosarcoma (10). These results indicate that caffeine-assisted multi-agent chemotherapy is superior to conventional multi-agent chemotherapies for the treatment of osteosarcoma, but the molecular mechanism of caffeine in this disease has not yet been fully elucidated.

Caffeine, a purine alkaloid, is a key component of many popular drinks, most notably tea and coffee, and is well known as a stimulatory agent because of its excitatory effects on nervous/humoral regulation. Methylxanthines, including caffeine and theophylline, increase cAMP by inhibiting PDE. We showed that caffeine increased phosphorylation of VASP, indicating elevated intracellular cAMP levels, and also increased the activity of PTEN while decreasing Akt activity in osteosarcoma cells, similarly to the general PDE inhibitor IBMX (Fig. 2). We observed that forskolin, an adenylate cyclase stimulator, reinforced the effects of caffeine on increasing intracellular cAMP and PTEN activity, and decreasing Akt activity in osteosarcoma cells (Fig. 3). These results suggested that production of cAMP by caffeine might regulate PTEN and Akt activity. Canetti *et al*. (26) reported that the exchange protein activated by cAMP (Epac)-1 agonist induces an increase in PTEN lipid phosphatase activity. Increased intracellular cAMP levels activate Epac-1, in turn causing an increase in SHP-1 phosphatase activity that is responsible for PTEN activation. Additional experimentation will be needed to define the mechanism of cAMP effects on PTEN activation in greater detail.

Akt is activated by PIP3, which is produced by PI3K and reduced by PTEN. Inactivation of Akt by caffeine suggests two possibilities: activation of PTEN or inactivation of PI3K. Nomura *et al.* (27) reported that caffeine suppressed phosphorylation and activation of Akt without inhibiting PI3K activation, thereby supporting our conclusion that caffeine-induced activation of PTEN inactivates Akt. To confirm that PTEN participated in

the effect caused by caffeine, PTEN was depleted by siRNA. In PTEN-knockdown cells, caffeine-mediated inactivation of Akt was markedly weakened (Fig. 1-D). This result supports an important role for PTEN in caffeine-induced antitumor effects.

Caffeine has also been shown to sensitize tumor cells to ionizing radiation and other genotoxic agents (28, 29). Caffeine inhibits ATM and ATR, proximal components of induced DNA damage, and cell cycle checkpoint pathways. ATM and ATR inhibit the passage of DNA-damaged cells from G2 to M phase, causing increased genomic instability. These results are supported by the findings that caffeine increases radiosensitivity, UVB-induced skin carcinogenesis, and sensitivity to chemotherapeutic agents. Moreover, some reports indicate that caffeine independently induces cellular apoptosis (29-31). Consistent with previous studies, the present study showed that caffeine induces apoptosis in osteosarcoma and fibrosarcoma cells (Fig. 1 and 4). Although caffeine is known to affect p53, Bax, Akt, and other apoptosis-related proteins (3), there have been no reports about caffeine's effect on the tumor suppressor protein, PTEN. In this study, we showed that PTEN is involved in caffeine-induced antitumor effects.

Work by He *et al.* (3) indicated that low concentrations of caffeine induced p53-dependent apoptosis via the Bax and caspase 3 pathways but this response was not induced in $p53^{-/-}$ cells. PTEN is believed to control p53 protein stability, both in a phosphatase-dependent and phosphatase-independent manner through interactions with p53 (32, 33). Furthermore, PTEN-p53 interactions have been demonstrated to enhance p53 DNA binding to endogenous p21 promoters (32, 33). Taken together, these results suggest the possibility that caffeine stabilizes and activates p53 through the activation of PTEN.

Our results demonstrated that caffeine enhances the apoptosis-inducing cytotoxicity

of cisplatin (Figure 6). Cisplatin increased the level of cleaved caspase 3, and this response was enhanced by caffeine. Thus, this mechanism likely explains the caffeine-assisted chemotherapeutic outcome in patients at our institutes.

In summary, our data provide novel insights into the mechanism by which caffeine induces apoptosis in osteosarcoma and fibrosarcoma cells. We demonstrate for the first time that caffeine activates PTEN, which antagonizes the stimulatory effects of PI3K in osteosarcoma cells and fibrosarcoma cells. This limits PI3K signaling, resulting in a suppression of downstream activation pathways such as Akt which are critical for anti-apoptotic functions. Our data demonstrate that these effects on PTEN are mediated by cAMP. These findings provide fundamental new insights into the regulation of PTEN-dependent signaling, and clarify one of the mechanisms of apoptosis induction by caffeine.

Acknowledgments

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Figure legends

Figure 1. Caffeine inhibits proliferation and decreases the level of PTEN and Akt phosphorylation in MG63 cells. MG63 cells were cultured in 100 µl 5% FBS/RPMI in 96-well plates (1×10^3/well). After 24 h, cells were exposed to 0-5 mM caffeine for 72 h. Results were determined with the cell proliferation assay. Data were calculated as a percentage of untreated controls. Caffeine decreased proliferation of MG63 cells in a dose-dependent manner **(A)**. **p<0.01. MG63 cells were cultured in 2 ml 5% FBS/RPMI (1×10^5/35 mm dish). After 24 h incubation, the cells were treated with 1 mM, or 5 mM caffeine for 30 min. Cells were disrupted with lysis buffer and expression of phospho-PTEN, total-PTEN, phospho-Akt, and total-Akt was measured by Western blotting. Caffeine decreased phosphorylated PTEN **(B)** and decreased phosphorylated Akt **(C)** in a dose-dependent manner. These results suggest that caffeine inhibits cell proliferation and increases PTEN activation and Akt inactivation.

$PTEN^+$ and PTEN-knockdown (generated with siRNA) MG63 cells (1×10^5) were suspended in 0.5% FBS/RPMI for 24 h and then treated with 5 mM caffeine for 30 min. Cells were disrupted with lysis buffer and levels of phospho-Akt, total-Akt, and total-PTEN were measured by Western blotting. The level of phospho-Akt was decreased by caffeine in the control cells, but the decrease was reduced in PTEN knockdown cells **(D)**. Thus, PTEN participates in caffeine-mediated Akt inactivation. Data are expressed as a mean ± SEM of independent three experiments. *P<0.05, **P<0.01 vs untreated controls.

Figure 2. Caffeine and IBMX phosphorylate VASP and decrease MG63 cell proliferation. As in Figure 1A, MG63 cells were treated with 0.25-5 mM caffeine, or 1 mM IBMX for 72 h. Then, cell proliferation was analyzed with the cell proliferation assay. Caffeine and IBMX

decreased proliferation of MG63 cells **(A)**. MG63 cells were incubated in RPMI without serum for 24 h, and the cells were treated with 5 mM caffeine, or 1 mM IBMX for 30 min. Cellular extracts were immunoblotted using anti-phospho-VASP, anti-β-actin, anti-phospho-PTEN, anti-PTEN, anti-phospho-Akt, and anti-Akt antibodies. As in Figure 1B and C, caffeine and IBMX increased the phosphorylation of VASP **(B)** and reduced phosphorylation of PTEN and Akt **(C, D)**. Therefore, caffeine and IBMX increase the level of cAMP, resulting in PTEN activation and Akt inactivation. Data are expressed as a mean ± SEM of independent three experiments. *p<0.05, **p<0.01 vs untreated controls.

Figure 3. Forskolin enhances PTEN activation and Akt inactivation by caffeine and IBMX in MG63 cells. MG63 cells were cultured in 2 ml 5% FBS/RPMI (1×10^5/35 mm dish). After 24 h incubation, the cells were treated with a low dose of forskolin (0.1 mM) alone, or combined with a low dose of caffeine (0.5 mM) and/or IBMX (0.1 mM) for 30 min. The expression of phospho-VASP, phospho-PTEN, and phospho-Akt was measured by Western blotting. Data are expressed as a mean ± SEM of independent three experiments. **P<0.01 vs untreated controls. Levels of phospho-VASP were slightly increased by forskolin, and markedly increased by combining with caffeine or IBMX **(A)**. The Levels of phospho-PTEN and phospho-Akt were markedly decreased by combining forskolin with caffeine or IBMX **(B, C)**. Thus, cAMP participates in caffeine-mediated PTEN activation and Akt inactivation.

Figure 4. Caffeine reduces growth and decreases phosphorylation of PTEN and Akt in HT1080 cells. As in Figure 1, caffeine reduced growth of HT1080 cells in a dose-dependent manner **(A)** and reduced phosphorylation of PTEN and phosphorylation of Akt, and increased phosphorylation of VASP **(B)** in HT1080 cells. **p<0.01. Thus, caffeine reduces proliferation via PTEN activation and Akt inactivation in both osteosarcoma cells and other sarcoma cells.

Figure 5. Caffeine enhances growth inhibition and pro-apoptotic pathway activation in MG63 cells exposed to cisplatin. As in Figure 1, MG63 cells were cultured in 96 well plates (1×10^3/well) and treated with or without 2 μg/ml cisplatin, followed by incubation with a low dose of caffeine (0.5 mM; similar to the concentration in human treatments) for 72 h. Then, cell proliferation was determined by cell proliferation assay **(A)**. Cells treated with 0.5 mM caffeine, or 2 μg/ml cisplatin had decreased amount of proliferation, which markedly decreased upon treatment of cells with both cisplatin and caffeine. Next, 1×10^5 MG63 cells were cultured in 35 mm dishes (1×10^5/dish) for 24 h and then exposed to either 0.5 mM caffeine, or 2 μg/ml cisplatin, or both. The level of phospho-PTEN, phospho-Akt, cleaved caspase 3, and β-actin was determined by Western blot analysis **(B)**. Cisplatin decreased the level of p-Akt and p-PTEN, and increased the level of cleaved caspase 3. Caffeine enhanced cisplatin-induced apoptosis. Thus, caffeine enhances the cytotoxicity of anticancer agents. **$p<0.01$.

Figure 6. Schematic diagram showing how caffeine induces apoptosis in osteosarcoma cells. Akt promotes cell proliferation and survival by inactivating apoptosis-inducing molecules. Akt activation is counter balanced by PTEN activity, which provides a negative regulation of PI3K signaling and reduces the level of PIP3 available for Akt recruitment. Our data indicate that caffeine activates PTEN through the elevation of intracellular cAMP, resulting in inhibition of Akt.

PDE: phosphodiesterase; AC: adenylate cyclase; PKA: protein kinase A; VASP: vasodilator stimulated phosphoprotein; Epac-1: exchange protein activated by cAMP-1; SHP-1: SH2-containing phosphatase 1; PIP2: phosphatidylinositol 3,4-bisphosphate; PIP3: phosphatidylinositol 3,4,5-trisphosphate; PI3K: phosphoinositide 3 kinase

Fig. 1

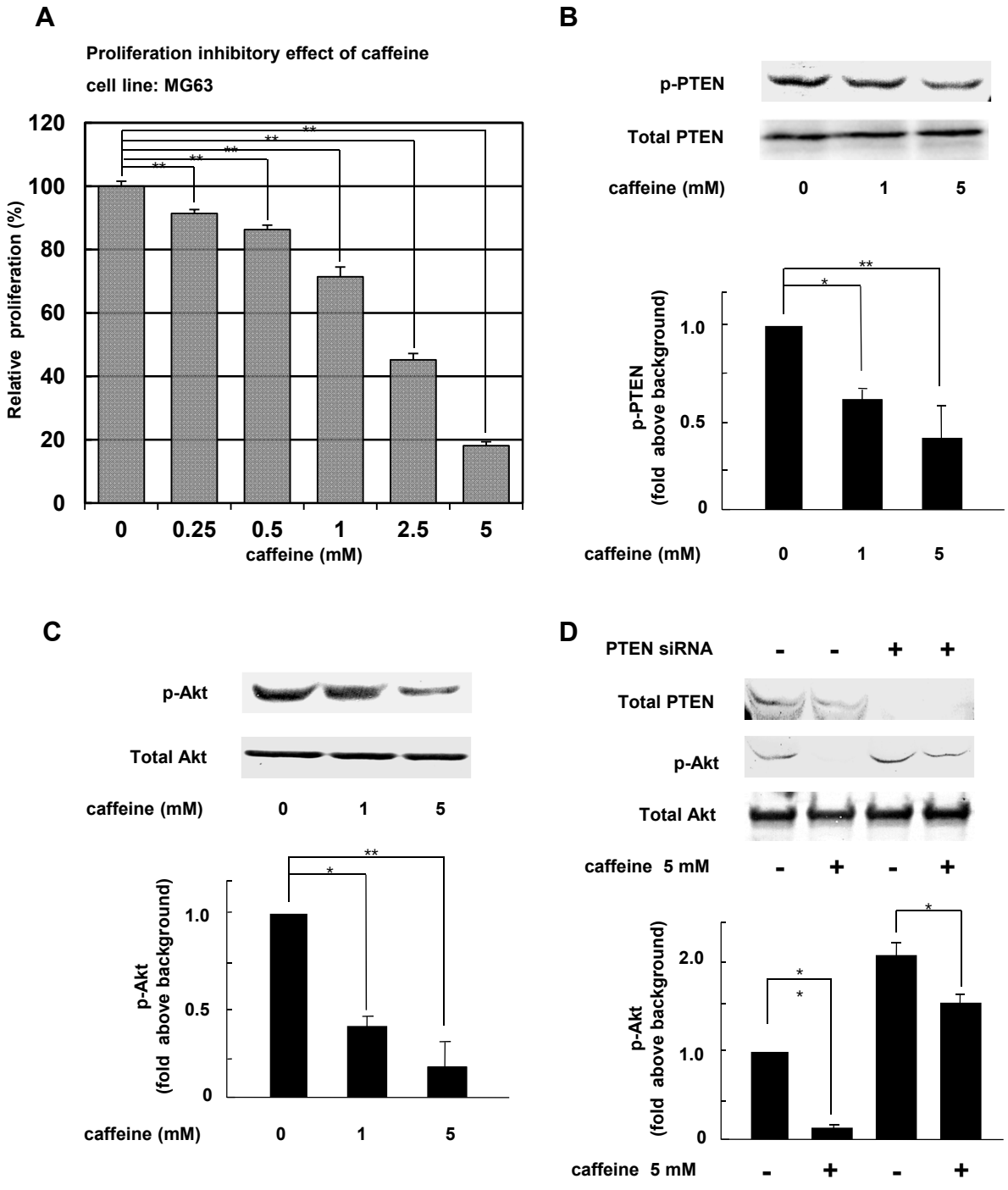

A
Proliferation inhibitory effect of caffeine
cell line: MG63
Relative proliferation (%)
120
100
80
60
40
20
0
0
0.25
0.5
1
2.5
5
caffeine (mM)
B
p-PTEN
Total PTEN
caffeine (mM) 0 1 5
p-PTEN (fold above background)
1.0
0.5
0
caffeine (mM) 0 1 5
C
p-Akt
Total Akt
caffeine (mM) 0 1 5
p-Akt (fold above background)
1.0
0.5
0
caffeine (mM) 0 1 5
D
PTEN siRNA - - + +
Total PTEN
p-Akt
Total Akt
caffeine 5 mM - + - +
p-Akt (fold above background)
2.0
1.0
0
caffeine 5 mM - + - +

Fig. 2

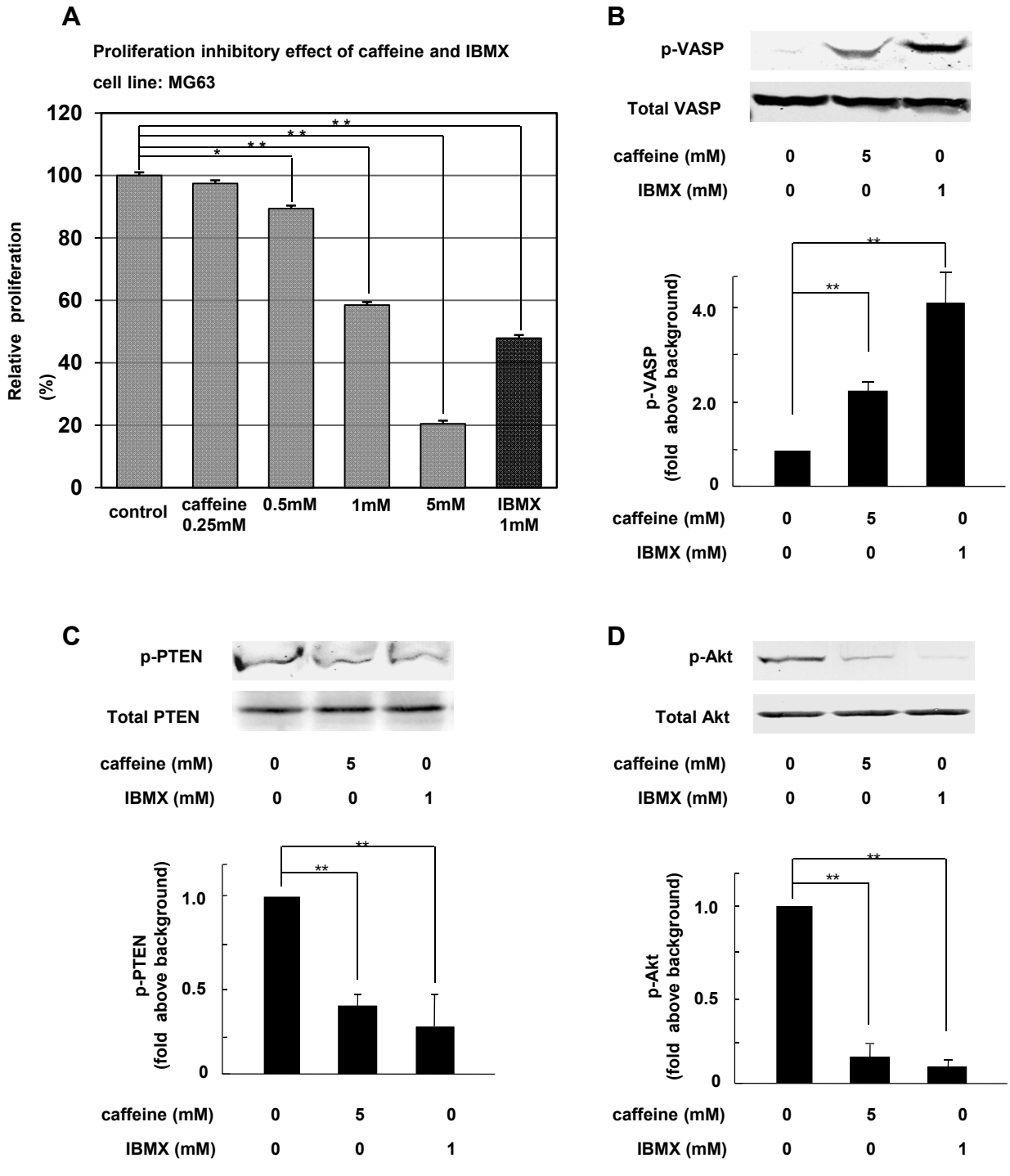

A
Proliferation inhibitory effect of caffeine and IBMX
cell line: MG63
Relative proliferation (%)
120
100
80
60
40
20
0
control
caffeine 0.25mM
0.5mM
1mM
5mM
IBMX 1mM
B
p-VASP
Total VASP
caffeine (mM) 0 5 0
IBMX (mM) 0 0 1
p-VASP (fold above background)
4.0
2.0
0
C
p-PTEN
Total PTEN
caffeine (mM) 0 5 0
IBMX (mM) 0 0 1
p-PTEN (fold above background)
1.0
0.5
0
D
p-Akt
Total Akt
caffeine (mM) 0 5 0
IBMX (mM) 0 0 1
p-Akt (fold above background)
1.0
0.5
0

Fig. 3

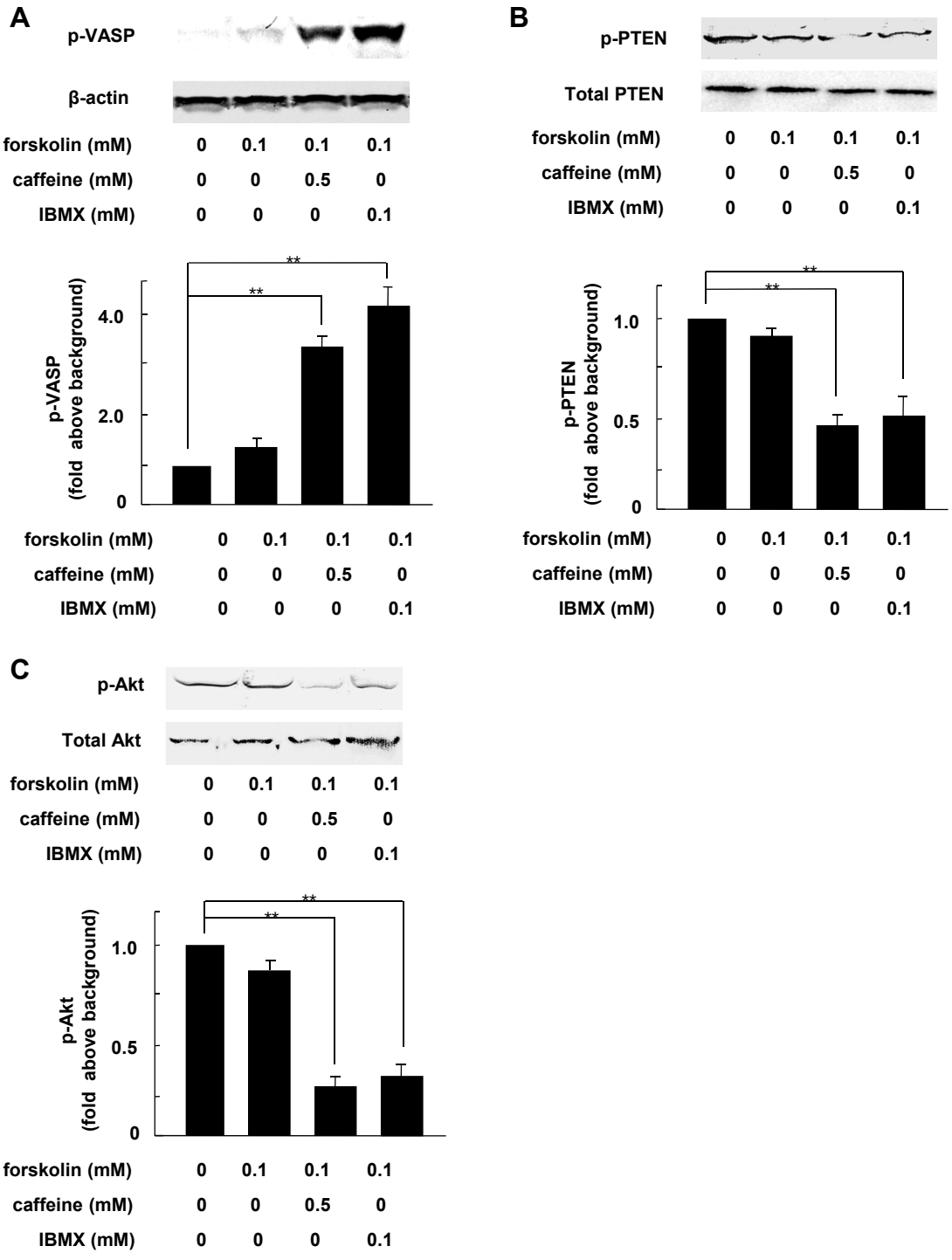
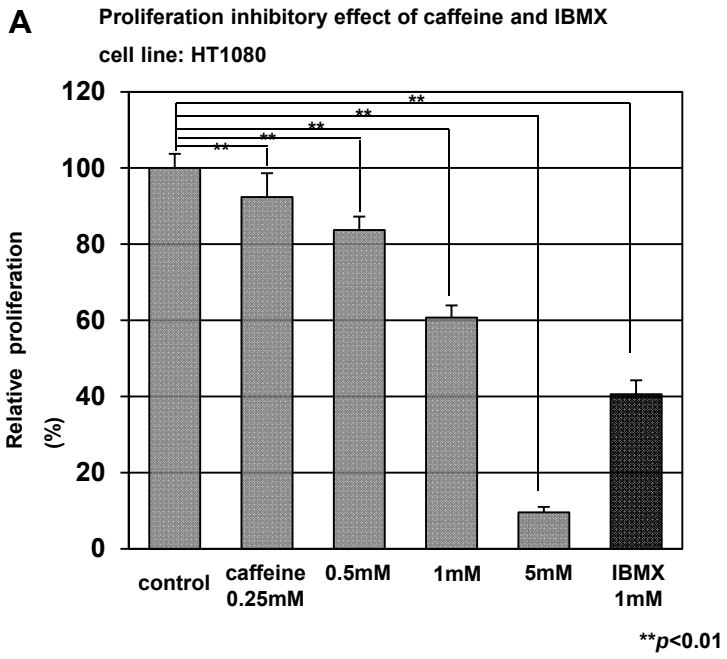

A
p-VASP
β-actin
forskolin (mM) 0 0.1 0.1 0.1
caffeine (mM) 0 0 0.5 0
IBMX (mM) 0 0 0 0.1
p-VASP (fold above background)
4.0
2.0
0
**
**
forskolin (mM) 0 0.1 0.1 0.1
caffeine (mM) 0 0 0.5 0
IBMX (mM) 0 0 0 0.1
B
p-PTEN
Total PTEN
forskolin (mM) 0 0.1 0.1 0.1
caffeine (mM) 0 0 0.5 0
IBMX (mM) 0 0 0 0.1
p-PTEN (fold above background)
1.0
0.5
0
**
**
forskolin (mM) 0 0.1 0.1 0.1
caffeine (mM) 0 0 0.5 0
IBMX (mM) 0 0 0 0.1
C
p-Akt
Total Akt
forskolin (mM) 0 0.1 0.1 0.1
caffeine (mM) 0 0 0.5 0
IBMX (mM) 0 0 0 0.1
p-Akt (fold above background)
1.0
0.5
0
**
**
forskolin (mM) 0 0.1 0.1 0.1
caffeine (mM) 0 0 0.5 0
IBMX (mM) 0 0 0 0.1

Fig. 4


A
Proliferation inhibitory effect of caffeine and IBMX
cell line: HT1080
Relative proliferation (%)
120
100
80
60
40
20
0
**
control
caffeine 0.25mM
0.5mM
1mM
5mM
IBMX 1mM
**p<0.01

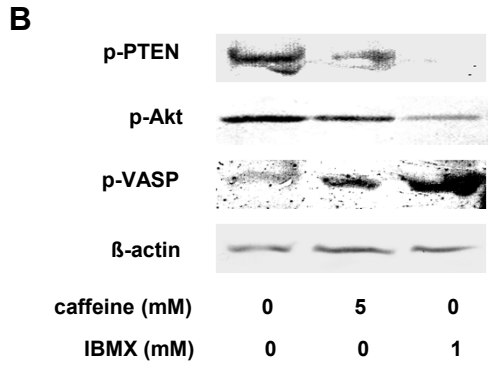
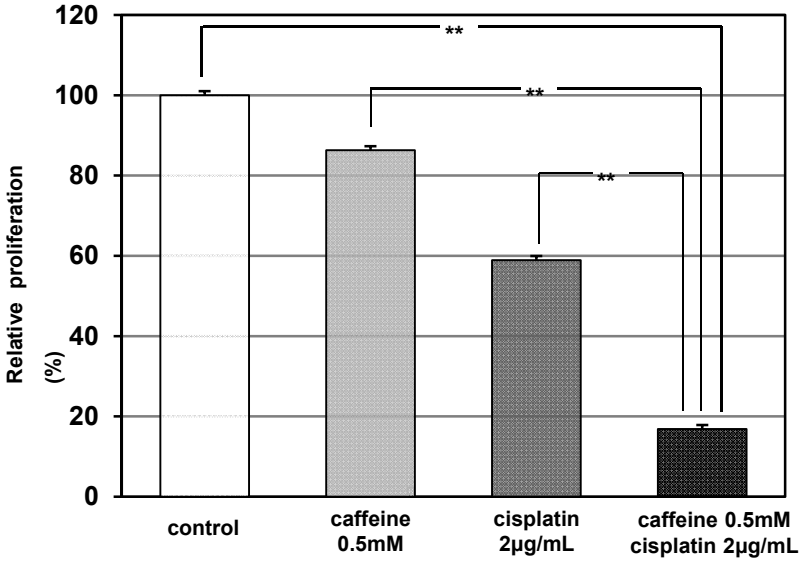

B
p-PTEN
p-Akt
p-VASP
ß-actin
caffeine (mM) 0 5 0
IBMX (mM) 0 0 1

Fig. 5

A


Proliferation inhibitory effect of caffeine and cisplatin
cell line: MG63
Relative proliferation (%)
120
100
80
60
40
20
0
**
**
**
control
caffeine 0.5mM
cisplatin 2μg/mL
caffeine 0.5mM cisplatin 2μg/mL

B

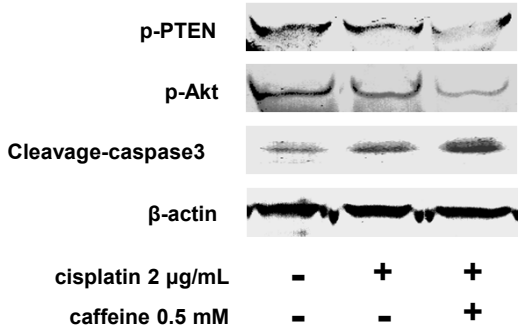
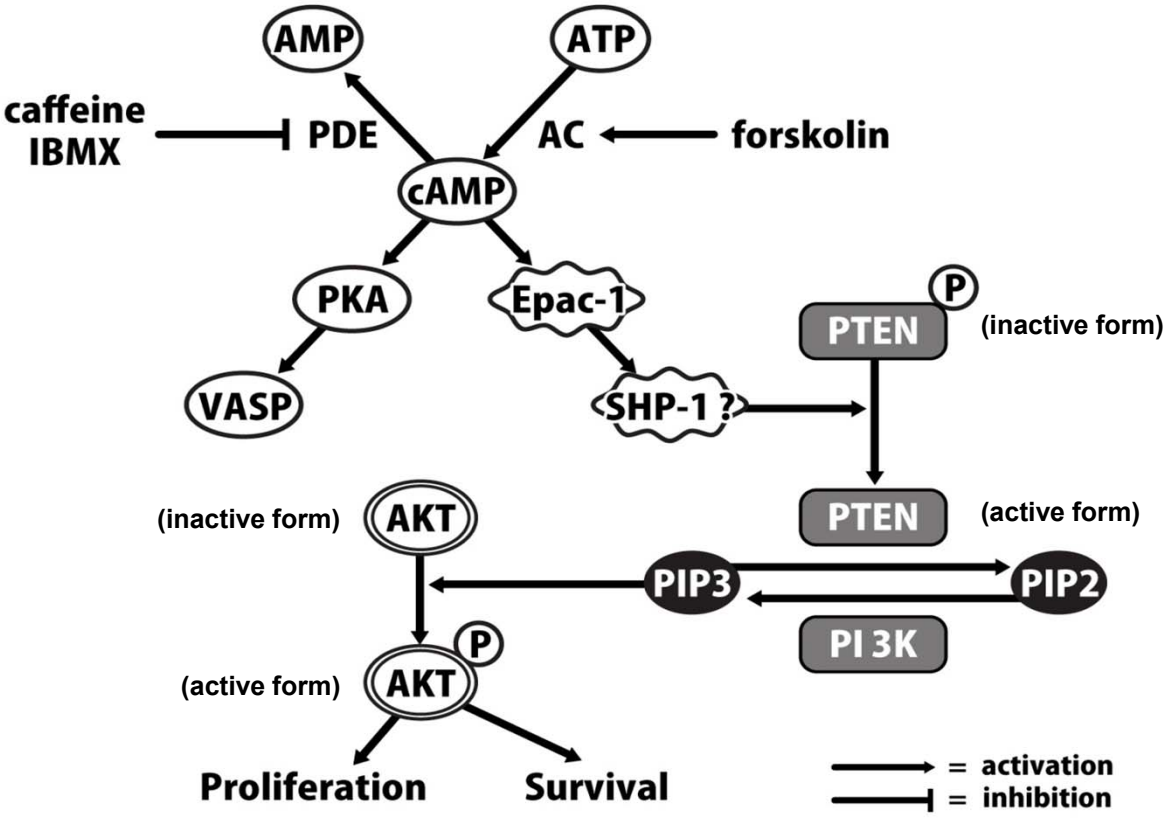

p-PTEN
p-Akt
Cleavage-caspase3
β-actin
cisplatin 2 μg/mL - + +
caffeine 0.5 mM - - +

Fig. 6


AMP
ATP
caffeine
IBMX
PDE
AC
forskolin
cAMP
PKA
Epac-1
P
PTEN
(inactive form)
VASP
SHP-1 ?
PTEN
(active form)
(inactive form)
AKT
PIP3
PIP2
PI 3K
P
(active form)
AKT
Proliferation
Survival
= activation
= inhibition