

Caffeine Induces Apoptosis of Osteosarcoma Cells by Inhibiting AKT/mTOR/S6K, NF- κ B and MAPK Pathways

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Abstract. We previously reported that caffeine-assisted chemotherapy improved the treatment of malignant bone and soft tissue tumours such as osteosarcoma. Caffeine affects tumour cells through various pathways, including phosphatase and tensin homolog deleted on chromosome 10 (PTEN), AKT, Bcl-2-associated X protein (BAX), caspase-3 and p53, and has therefore been indicated as being useful for the treatment of malignant tumours. Here, the effects of caffeine on the proliferation of HOS osteosarcoma cells were assessed by WST-8 assay, and the effects on the nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B), mammalian target of rapamycin (mTOR) and mitogen-activated protein kinase (MAPK) pathways were assessed by western blot analyses. Caffeine inhibited proliferation of HOS cells and suppressed NF- κ B, AKT, mTOR/S6K and ERK activities. Our results support those from previous studies relating to the use of caffeine in the treatment of osteosarcoma.

Osteosarcoma is the most common primary malignant bone tumour 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 (1). At our institute, caffeine-assisted multi-agent chemotherapy has improved the treatment success of malignant bone and soft tissue tumours such as osteosarcoma (2). While conventional multi-agent chemotherapies yield a local tumour response of

approximately 63% and a five-year survival of 67.5% for non-metastatic osteosarcoma (1), the response rate of caffeine-assisted chemotherapy is over 80% and the five-year survival rate is 90% (five-year event-free rate, 76%) for primary non-metastatic osteosarcoma (2). These results indicate that caffeine-assisted multi-agent chemotherapy is superior to conventional multi-agent chemotherapies in the treatment of osteosarcoma.

Several mechanisms have been proposed to explain how caffeine enhances chemosensitivity and induces apoptosis of tumour cells. 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 (3). Many anticancer agents directly induce DNA damage, and cell cycle checkpoints are activated to repair the damaged DNA. Caffeine inhibits DNA repair by inactivation of the cell cycle checkpoints, resulting in cancer cell death. Conversely, caffeine also independently inhibits the proliferation of, and induces apoptosis of tumour cells. He *et al.* (4) indicated that a low concentration of caffeine induced p53-dependent apoptosis via the Bcl-2-associated X protein (BAX) and caspase-3 pathways. We previously reported that a moderate concentration of caffeine activated PTEN, which antagonizes the stimulatory effects of PI3K in sarcoma cells (5). Saiki *et al.* (6) reported that a high concentration of caffeine activates the extracellular-signal regulated (ERK) and mitogen-activated protein kinase (MAPK) pathways.

There are many pathways that regulate the apoptosis, survival, proliferation and migration of tumour cells. The mammalian target of rapamycin kinase (mTOR) is activated by AKT, and regulates cell survival, migration and proliferation. Nuclear factor-kappa B (NF- κ B) is a transcription factor that plays a pivotal role in the control of inflammation, cell proliferation, metastasis and apoptosis (7, 8).

In this study, we investigated the effects of caffeine on AKT/mTOR, ERK, and NF- κ B pathways in osteosarcoma cells.

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Materials and Methods

Reagents and antibodies. RPMI 1640 medium supplemented with L-glutamine, phenol red and caffeine was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan); foetal bovine serum (FBS) was obtained from Sigma (St. Louis, MO, USA) and penicillin and streptomycin from Gibco-Invitrogen (Carlsbad, CA, USA). Rapamycin was purchased from Merck (Darmstadt, Germany).

Anti-phospho-specific mTOR (Ser2448), anti-phospho-specific p70 S6K (Thr389), anti-phospho-specific p70 S6K (Ser371), anti-phospho-specific 4E-BP1 (Thr37/46), anti-phospho-specific p44/42 MAPK (Thr202/Tyr204), anti-phospho-specific NF- κ B p65 (Ser536), anti- β -actin, horseradish peroxidase (HRP)-linked anti-rabbit IgG, anti-phospho-specific ERK 1/2 and anti-phospho-specific AKT (Ser473) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA).

Cell culture. The HOS human osteosarcoma cell line was obtained from the American Type Culture Collection (Manassas, VA, USA) and was maintained in RPMI 1640 supplemented with 10% FBS, penicillin and streptomycin at 37°C in 5% CO₂.

Cell proliferation assay. Cell proliferation was analysed with Cell Counting Kit 8 (Dojindo, Japan). HOS cells were seeded in 96-well plates at a density of 1×10^3 cells/well. After 24 h incubation, the cells were treated or not treated with 1-5 μ M rapamycin or 0.5-5 mM caffeine for 72 h. The cells were then incubated with 10 μ M WST-8 for 2 h. Absorbance of the coloured formazan product produced by the mitochondrial dehydrogenases in metabolically active cells was recorded at 450 nm as the background value. Cell proliferation was expressed as a percentage of the absorbance obtained in treated wells relative to that in untreated control wells. The concentrations of rapamycin and caffeine corresponding to 50% cell death were used as IC₅₀ values.

S-6K, p44/42 MAPK (ERK), AKT and NF- κ B activity assays. To determine the effect of caffeine and rapamycin on mTOR, S-6K, ERK, AKT and NF- κ B activities, we investigated the levels of phospho-mTOR, phospho-S-6K, phospho-ERK, phospho-AKT and phospho-NF- κ B, respectively, in cells using various methods. The extent of the increases in these phosphorylated proteins is indicative of the extent of their signalling activation. HOS cells were incubated in RPMI-containing serum for 24 h, and then treated with 5 mM caffeine or 5 μ M rapamycin for 30 min. Cell extracts were immunoblotted using anti-phospho mTOR (Ser2448), anti-phospho p70 S6K (Thr389), anti-phospho p44/42 MAPK (Thr202/Tyr204), anti-phospho-AKT (Ser473) and anti-phospho-NF- κ B p65 (Ser536) antibodies.

Western blot analysis. Western blot analyses were performed as described previously (9, 10). Briefly, proteins were extracted from cells, and their concentrations were determined using a protein assay. Equal amounts of protein were separated using 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Resolved proteins were transferred onto polyvinylidene fluoride (PVDF) membranes, which were incubated with primary antibodies (1:1000), followed by incubation with HRP-linked secondary antibodies (1:2000). The blots were developed using Immobilon Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA, USA).

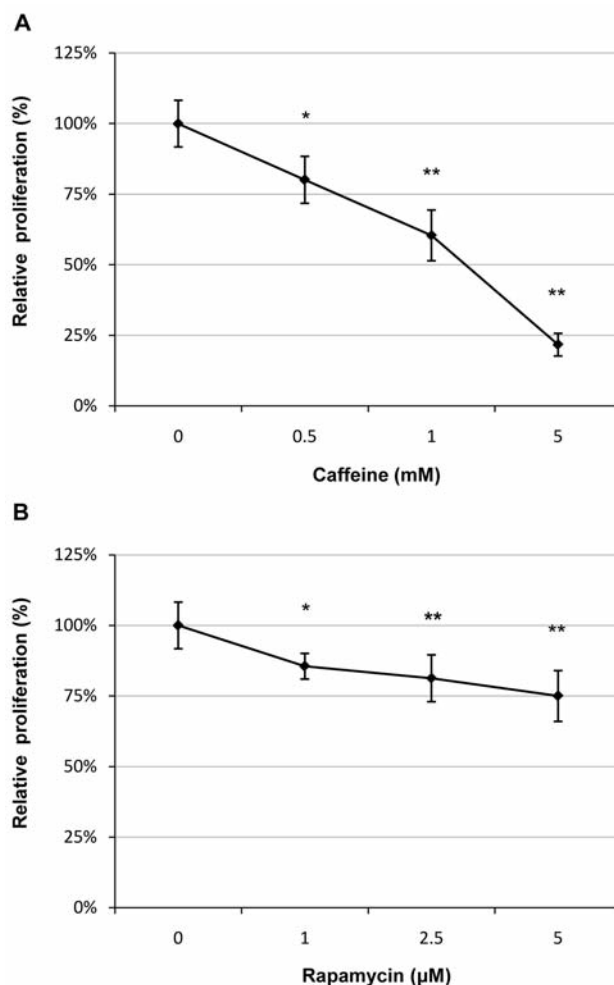


Figure 1. Caffeine and rapamycin inhibit proliferation of HOS osteosarcoma cells in a dose-dependent manner. HOS cells were cultured in 100 μ l 5% FBS/RPMI in 96-well plates (1×10^3 /well). After 24 h, cells were exposed to 0-5 mM caffeine or 0-5 μ M rapamycin for 72 h. Results were determined by cell proliferation assay. Data were calculated as a percentage of untreated controls. Caffeine reduces the proliferation of HOS cells in a dose-dependent manner (A). Data are expressed as the mean \pm SEM of three independent experiments. * $p < 0.05$ and ** $p < 0.01$ vs. untreated controls.

Statistical analysis. Densitometric analysis was performed using Image J 1.43 (National Institute of Mental Health, Bethesda, Maryland, USA) on immunoblots from three independent experiments. Data are presented as the mean \pm SEM from at least three independent experiments. Statistical analyses were performed using ANOVA, followed by Dunnett's test, and $p < 0.05$ was considered statistically significant.

Results

Caffeine and rapamycin inhibit proliferation of HOS cells in a dose-dependent manner. The proliferation of caffeine-

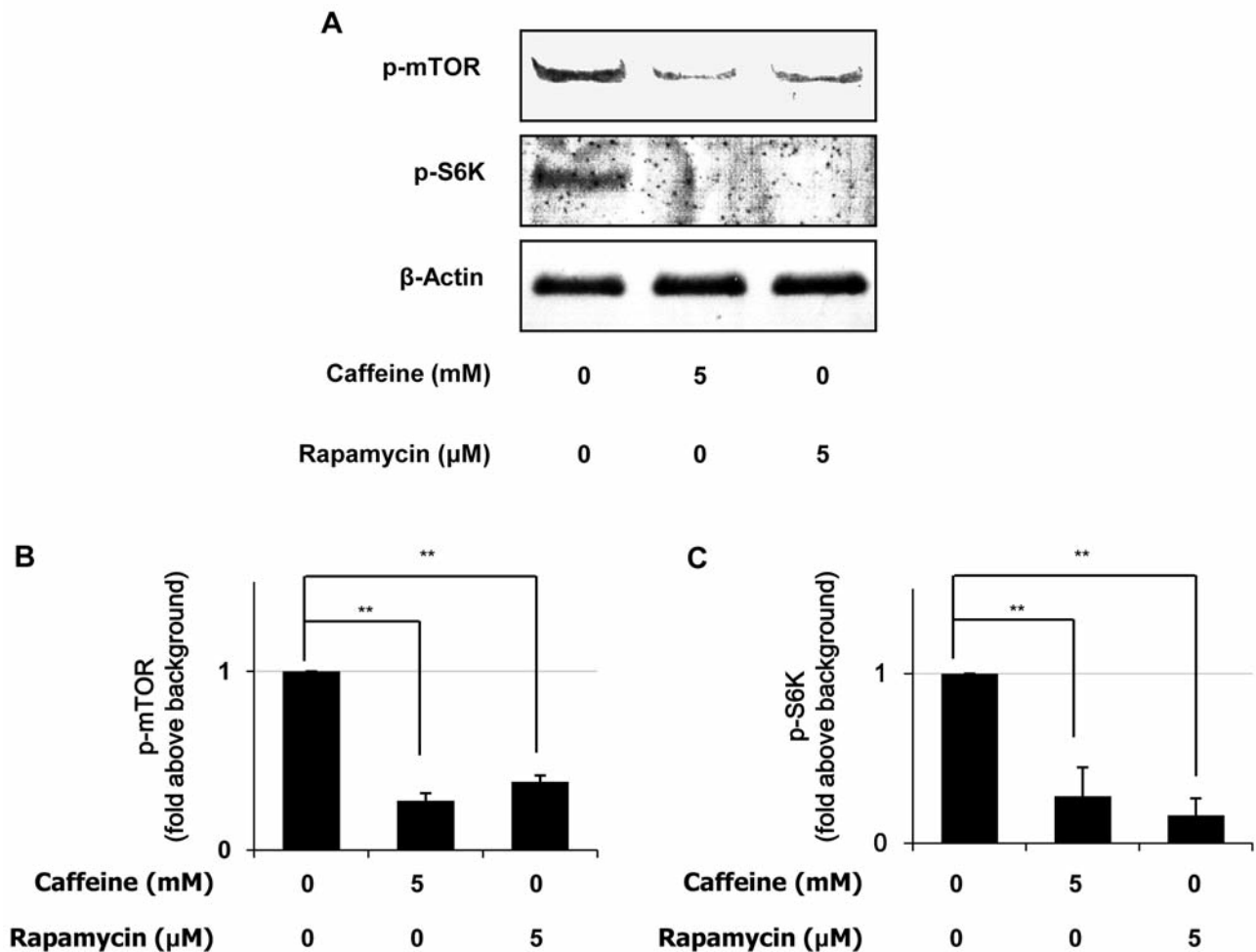


Figure 2. Caffeine and rapamycin reduce the amount of phosphorylated mTOR and S-6K in HOS osteosarcoma cells. HOS cells were cultured in 2 ml 5% FBS/RPMI (1×10^5 /35 mm dish). After 24 h incubation, the cells were treated with 5 mM caffeine or 5 μ M rapamycin for 30 min. The expression of phospho-mTOR and phospho-S6K was measured by western blotting (A). Proteins were quantified by Image J (B and C). Levels of phospho-mTOR and phospho-S6K were markedly reduced by caffeine and rapamycin. Data are expressed as the mean \pm SEM of three independent experiments. $**p < 0.01$ vs. untreated controls.

treated HOS cells decreased in a dose-dependent fashion ($IC_{50}=2.80$ mM) (Figure 1A). To determine the contribution of the mTOR pathway to the proliferation of HOS cells, rapamycin was used as a positive control because it is an inhibitor of the mTOR pathway. The proliferation of HOS cells treated with rapamycin decreased in a dose-dependent fashion ($IC_{50}=14.2$ μ M) (Figure 1B).

Caffeine and rapamycin reduce the amount of phosphorylated mTOR and S-6K in HOS cells. HOS cells were treated with 5 mM caffeine or 5 μ M rapamycin for 30 min. The expression of phospho-mTOR and phospho-S6K was analysed by western blotting. Levels of phospho-mTOR and phospho-S6K were reduced by caffeine and rapamycin

(Figure 2A-C). These results indicate that caffeine induces inactivation of mTOR and S-6K.

Caffeine reduces the amount of phosphorylated AKT in HOS cells. To confirm that caffeine suppresses the activation of AKT, phospho-AKT was analysed in HOS cells treated with 5 mM caffeine or 5 μ M rapamycin for 30 min. Caffeine reduced the level of phospho-AKT in HOS cells, while rapamycin did not (Figure 3A and B). This result indicates that caffeine inactivates AKT, and that rapamycin does not affect AKT activity. As shown in Figure 2, caffeine induced inactivation of mTOR and S-6K. These findings indicate that caffeine inactivates the AKT/mTOR pathway, although it is unclear whether caffeine directly inactivates mTOR and S6K.

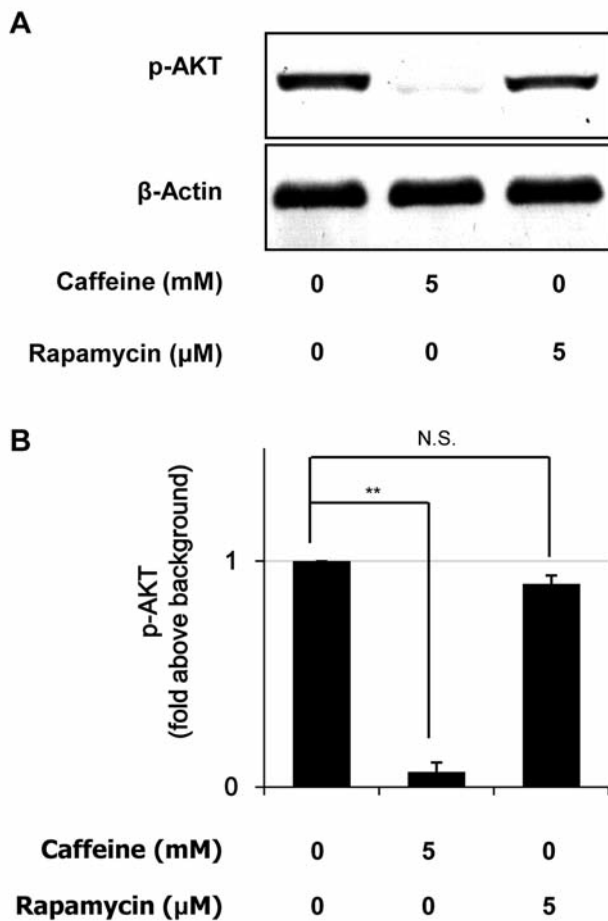


Figure 3. Caffeine reduces the amount of phosphorylated AKT in HOS osteosarcoma cells. HOS cells were incubated in 5% FBS/RPMI ($1 \times 10^5/35$ mm dish) for 24 h, and the cells were treated with 5 mM caffeine or 5 μM rapamycin for 30 min. Cellular extracts were immunoblotted using anti-phospho-AKT and anti-β-actin (A). Proteins were quantified by Image J (B and C). Caffeine reduced the phosphorylation of AKT. However, phosphorylation of AKT was unaffected by rapamycin. Data are expressed as mean \pm SEM of three independent experiments. ** $p < 0.01$ vs. untreated controls. N.S.: Not significantly different from the control.

Caffeine reduces the amount of phosphorylated ERK and NF-κB in HOS cells. To determine the effects of caffeine on the IκB kinase (IKK)/NF-κB and ERK/MAPK pathways, phospho-ERK and phospho-NF-κB were analysed in HOS cells treated with 5 mM caffeine or 5 μM rapamycin for 30 min. Caffeine reduced the levels of phospho-ERK and phospho-NF-κB in HOS cells (Figure 4A). However, rapamycin did not affect the levels of phospho-ERK and phospho-NF-κB in HOS cells (Figure 4A). These findings suggest that caffeine inactivates NF-κB, and that the effect is independent of the mTOR/S6K pathway.

Discussion

Caffeine, a methylxanthine derivative, increases cAMP by inhibiting phosphodiesterase (PDE) activity, and it is known to have diuretic and central nervous system-stimulatory effects. On the other hand, caffeine is an inhibitor of ATM and ATR kinases, which are master regulators of DNA damage-induced cell cycle checkpoints (3, 11). Therefore, caffeine has both anticancer effects and the ability to enhance the cytotoxic activity of anticancer drugs by inhibiting DNA repair pathways (4, 12, 13). We developed a caffeine-assisted chemotherapy for bone and soft tissue sarcoma, and while the five-year survival rate of patients with osteosarcomas is generally 60-70% (1, 14, 15), we previously reported that caffeine-assisted chemotherapy induced a complete response in more than 80% of patients with osteosarcoma (16), and increased the five-year survival rate by approximately 90% (2). We have also reported the use of caffeine-assisted chemotherapy for other malignant bone and soft tissue tumours (17-20).

A few of the molecular mechanisms by which caffeine inhibits tumour progression have been elucidated. In response to various genotoxic stresses, such as anticancer drugs and irradiation, cell cycle checkpoints are inactivated and proliferating cells undergo G-2 phase arrest, which provides sufficient time for DNA repair in order to maintain genomic integrity (21). Caffeine is known to overcome the chemotherapy/radiation-induced G-2 phase arrest by inhibiting G-2 checkpoint activity, resulting in reduced time for repair of damaged DNA and eventually apoptosis (3). He *et al.* (4) reported that a low concentration of caffeine induced p53-dependent apoptosis through the BAX and caspase-3 pathways. Caffeine does not affect radiosensitivity in normal cells, but induces increased radiosensitivity in hepatocellular cancer cells (22). Caffeine has also been shown to inhibit PI3K and components of the PI3K/AKT pathway (23). We reported that caffeine activates PTEN and inactivates AKT, resulting in apoptosis of tumour cells (5). These results suggest that caffeine affects various pathways that control the survival and proliferation of cancer cells.

This study showed that 5 mM caffeine inhibited the AKT/mTOR/p70S6K, NF-κB and ERK1/2 pathways in HOS cells. Notably, to our knowledge, this is the first report on caffeine inhibition of the IKK/NF-κB pathway. NF-κB is a transcription factor that plays a pivotal role in the control of inflammation, cell proliferation and apoptosis (24). The NF-κB pathway is often constitutively activated in human cancer and much research has focused on identifying inhibitors of this pathway (25, 26). The PI3K/AKT pathway is critical for cell survival and is a well-known regulator of NF-κB activation (26). ERK/MAPK is another important pathway involved in cell survival (27) and has been shown to activate NF-κB responses by activating p90 RSK (28). Saiki *et al.* (6) reported that a high concentration of caffeine (10-25 mM)

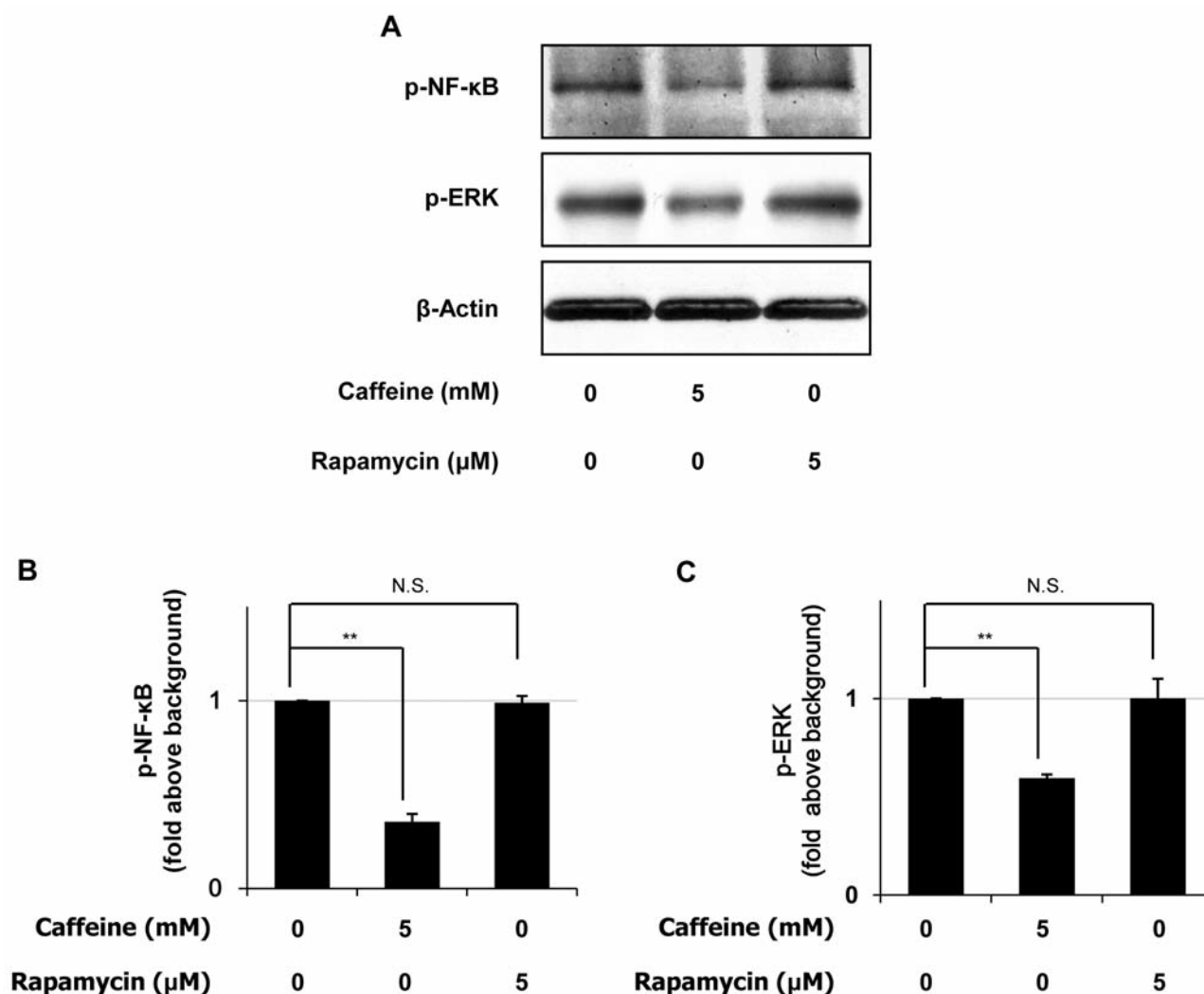


Figure 4. Caffeine reduces the amount of phosphorylated ERK and NF- κ B in HOS osteosarcoma cells. HOS cells were cultured in 2 ml 5% FBS/RPMI (1×10^5 /35 mm dish). After 24 h incubation, the cells were treated with 5 mM caffeine or 5 μ M rapamycin for 30 min. Cellular extracts were immunoblotted using anti-phospho-NF- κ B, anti-phospho-ERK, and anti- β -actin. Caffeine reduced the phosphorylation of NF- κ B and ERK. However, phosphorylation of NF- κ B and ERK was unaffected by the treatment of rapamycin. Data are expressed as the mean \pm SEM of three independent experiments. ** $p < 0.01$ vs. untreated controls. N.S.: Not significantly different from the control.

inhibited the Akt/mTOR/p70S6K pathway, activated the ERK1/2 pathway and induced autophagy. On the other hand, our data showed that caffeine has an inhibitory effect on ERK. The difference relating to the effects of caffeine may depend on the concentration used. Considering that the IC_{50} of caffeine is 2.7-2.8 mM (5), a moderate concentration of caffeine is thought to have an inhibitory effect on the ERK pathway.

The NF- κ B pathway has various effects on cell survival, apoptosis, angiogenesis, carcinogenesis and tumour progression (7). Given the effects of NF- κ B on tumour development and progression, this factor and the signalling pathways involved in its activation are attractive targets for cancer prevention and

therapy. Furthermore, the fact that caffeine inhibits NF- κ B and the proliferation of tumour cells supports the prospects of an NF- κ B inhibitor for cancer therapy.

In conclusion, our data reveals various mechanisms by which caffeine induces apoptosis of osteosarcoma cells. In particular, we demonstrated for the first time that caffeine inhibits NF- κ B, which plays an important role in tumourigenesis and tumour progression.

Acknowledgements

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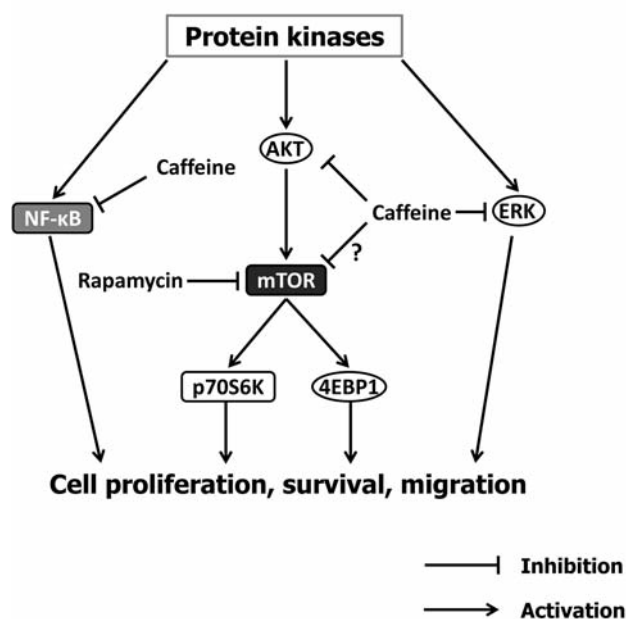


Figure 5. Schematic diagram showing how caffeine inhibits proliferation of HOS osteosarcoma cells. NF- κ B is a transcription factor that plays a pivotal role in the control of inflammation, cell proliferation and apoptosis. The AKT/mTOR pathway is critical for cell survival and is a well-known regulator of NF- κ B activation. ERK is also an important pathway involved in cell survival. Although rapamycin inactivates only the mTOR pathway, caffeine inactivates the ERK, NF- κ B and AKT/mTOR pathways.

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