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Blockade of AKT activation in prostate cancer cells with a small molecule inhibitor, 9-chloro-2-methylellipticinium acetate (CMEP)☆

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Abbreviations:
AR, androgen receptor
CMEP,
9-chloro-2-methylellipticinium acetate
GSK-3, glycogen synthase kinase-3
MAPK, mitogen-activated kinases
mTOR,
mammalian target of rapamycin
MTT, 3-[4,5-dimethythiazol-2-yl]2, 5-diphenyltetrozolium bromide
PI3K, phosphatidylinositol 3-kinase
PKB, protein kinase B
PTEN,
phosphatase and tensin homolog
TSC, tubererous sclerosis complex

ABSTRACT

AKT inhibitors are potentially promising drug candidates for the treatment of cancer. The inhibitory effects of a potent and selective AKT/BKB small molecule inhibitor, 9-chloro-2-methylellipticinium acetate (CMEP), on the activation of AKT, its antiproliferation and apoptosis-inducing effects in prostate cancer cell lines: DU-145, PC-3, LNCaP, and CL-1, an androgen-independent LNCaP variant, and CL-1 xenograft mouse model were assessed by Western blot analysis, kinase assay, cell survival assay, and apoptosis assay in this report. It has been observed that the expression levels of AKT1, AKT2, and AKT3 vary, but the levels of phospho-Ser473 AKT and phospho-Thr308 AKT are quite unique in these cancer cell lines, and that CL-1 cells have the highest basal levels of AKT activation among these cell lines. In PC-3 cells, CMEP has been found to inhibit only AKT activation at both normal and serum-starvation conditions, not to inhibit PI3K, PDK1, or MAPK. More importantly, it has been discovered that CMEP inhibits cell proliferation, and induces apoptosis in prostate cancer cells which have high-levels of AKT activation and lack PTEN or harbor PTEN mutation, such as CL-1, LNCaP, and PC-3; only shows a minimal activity in DU-145 cancer cells which do not have AKT activation. Furthermore, it has been demonstrated that CMEP treatment inhibits phospho-Ser473 AKT and phospho-p70S6K while stimulating TSC2 in the tumor tissue from CL-1-bearing mice. In conclusion, by specific blockade of the activation of AKT, CMEP preferentially inhibits growth and induces apoptosis in prostate cancer cells which have high-levels of AKT activation.

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1. Introduction

AKT (AKT8 virus oncogene cellular homolog) or protein kinase B (PKB) is a serine/threonine protein kinase functioning downstream of phosphatidylinositol 3-kinase (PI3K) in response to mitogen or growth factor stimulation [1]. Three isoforms of AKT are present in mammalian cells: AKT1, AKT2, and AKT3 [2–4]. Phospho-Serine473 AKT is the major active form of AKT [5,6]. The tumor suppressor, PTEN (phosphatase and tensin homolog) negatively regulates the activity of AKT [7–11]. High-level of AKT activation has been associated with the development and metastasis of cancer [12–15]. More importantly, accumulating evidence has shown that this AKT activation contributes to the resistance of cancer cells to conventional therapies including chemotherapy, radiation therapy and hormone therapy [16–19]. AKT activation not only directly inhibits apoptosis, or programmed cell death, by multiple mechanisms involving inhibiting the conformational change of pro-apoptosis protein Bax [20], phosphorylation of several other components of the apoptotic machinery, including BAD [21] and caspase-9 [22]; but also modulates apoptosis indirectly by influencing the activities of several transcription factors, including fork head transcription factor (FKHR), NF-κB, and cyclic AMP-responsive element binding protein [5]. In addition, AKT activation results in the increase of mammalian cell size [23] and cell mass by stimulating protein synthesis as well as by inhibiting protein degradation [24], through activation of downstream protein synthesis signal transduction pathway via TSC1/TSC2 (tuberous sclerosis complex) [25–30].

Prostate cancer (CaP) is the most common malignancy and the second leading cause of cancer mortality in men. The development of CaP in humans has been viewed as a multistage process, involving the onset as small latent carcinoma of low histological grade to large metastatic lesion of higher grade. Unfortunately, there are limited treatment options available for this disease because chemotherapy and radiation therapy are largely ineffective, and metastatic disease frequently develops even after potentially curative surgery. In general, significantly increased AKT activation was detected in more than 50% of primary carcinomas of prostate, especially hormone-refractory prostate carcinoma [31], therefore direct inhibition of AKT activation may be an effective anticancer strategy.

Numerous studies have implicated the loss of PTEN in CaP progression [32]. The activity of AKT3 was detected to be 40–100-fold elevated in PC-3 cells that do not have the tumor suppressor PTEN in comparison to DU-145 cells that do have PTEN [33]. When injected orthotopically into the prostate of nude mice, PC-3 cells grow aggressively locally and readily metastasize to regional lymph nodes, but adenoviral infection of PTEN into PTEN-null PC-3 cell lines resulted in decreased metastatic potential without significantly altering tumor size via the predominant mechanism of G1 arrest but not apoptosis [32].

In men with metastatic CaP, androgen blockade is the only treatment available. Androgen ablation usually decreases the volume of the primary and metastatic lesions by inducing apoptosis. However, after this initial response, the tumors recur in an androgen-independent form that is unresponsive to additional androgen withdrawal and are resistant to cure by chemotherapy. The mechanism of CaP progression to hormone independence remains unclear [33]. AKT3 was found to be 20–60-fold higher in androgen-insensitive than in androgen-responsive CaP cells [34]. Sequence analysis of AR reveals two AKT consensus sequences (RXRXXS/T), located at the amino-terminal domain and carboxyl-terminal domain that may mediate the signal from HER-2/Neu-AKT pathway. It has been demonstrated that AKT phosphorylates androgen receptor (AR) at Ser-210, inhibits AR transactivation, and blocks AR-induced apoptosis [35]. On the other hand, in the prostate, AR is believed to stimulate the activities which antagonize apoptosis and induce cell proliferation [36]. According to a recent report, the presence of AR is essential for androgen-independent CaP cell proliferation [37]. In addition, the AR expression level increases in androgen-independent CaP [38]. It has been shown that there is a link between PI3K/AKT activation and the augmentation of AR transactivation through GSK3β (glycogen synthase kinase) phosphorylation and nuclear accumulation of β-catenin [39].

Although the reported frequencies of *p53* mutations in primary CaP remain controversial, most agree that mutations of *p53* are common in advanced CaP, suggesting that *p53* mutations may be involved in the progression of CaP [33]. There are some observations suggest that gain-of-function (GOF) p53 mutants mediate the androgen-independent growth of LNCaP cells without the involvement of AKT [33], but recent studies have shown that PI3K–AKT signaling promotes the phosphorylation and movement of the Mdm2 oncoprotein into the nucleus, where it down-regulates p53 [40–42].

Taken together, AKT plays important roles in promoting the development and metastasis of CaP cells. Direct inhibition of the activation of AKT may represent an attractive therapeutic strategy for androgen-insensitive CaP, which lacks functional PTEN.

There are several reports related to the inhibitory effects of natural compounds against AKT, like curcumin [43], deguelin [44], and indo-3-carbinol [45], but whether these compounds directly inhibit AKT and its underlining molecular mechanism remain unknown. Recently, several groups have reported the discoveries of AKT selective or AKT isoform-selective small molecule inhibitors [46–49], which all directly inhibit AKT, and shown therapeutic potential toward irradiating cancer cells with high-levels of AKT activation.

Using a bioinformatics-based approach, combined with AKT kinase activity assay, we identified a selective AKT small molecule inhibitor, 9-chloro-2-methylellipticinium acetate (CMEP). API-59cj-OME, one analog of CMEP, has been shown to preferentially inhibit growth and induce apoptosis in ovarian [50] and endometrial [51] cancer cells, which have high-levels of AKT activation and lack PTEN or harbor PTEN mutation. In this report, we present that CMEP inhibits growth and induces apoptosis in Cap cells with high-levels of AKT activation by selectively blocking the activation of AKT.

2. Materials and methods

2.1. Cell lines and reagents

Human prostate cancer cell lines DU-145, LNCaP, and PC-3 were purchased from the American Type Culture Collection

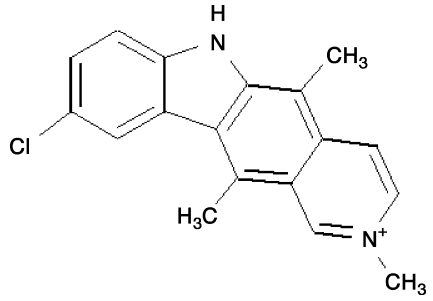

Fig. 1 – Chemical structure of 9-chloro-2-methylellipticinium acetate (CMEP).

(ATCC, Rockville, MD). Cells were maintained in Isokov's modified Eagles medium (Biofluids, Rockville) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Gibco, BRL) plus 1% glutamine (Gibco, BRL). CL-1 was originally obtained from Dr. Tso (UCLA School of Medicine), maintained in the same medium but with 10% charcoal-stripped fetal bovine serum [52]. All cell lines were incubated at 37 °C in 5% (v/v) CO_2. CMEP (NSC632855) was requested from Drug Synthesis & Chemistry Branch, National Cancer Institute, and dissolved in DMSO. Fig. 1 is the chemical structure of CMEP.

2.2. AKT kinase assay

A sensitive and quantitative cell-free and cell-based *in vitro* AKT kinase assay was established based on an AKT kinase kit from Cell Signaling (Beverly, MA). Briefly, prostate cancer cell lysates were harvested using 1× ice-cold cell lysis buffer, 20 μl of resuspended immobilized AKT antibody (monoclonal antibody 1G1, recognizes all three AKT isoforms) slurry was added to 200 μl lysates (1 μg/μl), and incubated with gentle rocking 2–3 h at 4 °C, then microcentrifuged for 30 s at 4 °C, and the pellet was washed once with 1× lysis buffer and 1× kinase buffer, respectively. AKT kinase reaction was carried out with the pellet resuspended with 1× kinase buffer supplemented with 200 μM of ATP, 1 μg of GSK-3 fusion protein (molecular wt.: 30 kDa. It is the crosstide of GSK-3α/β, corresponding to residues surrounding GSK-3α/β (Ser21/9) (CGPKGPGRRRTSSFAEG), was fused to the amino-terminus of paramysin for preparation purpose) and desired concentration of CMEP. The reaction was terminated after incubation at 30 °C for 30 min with 3× SDS sample buffer, subjected to 4–20% SDS-PAGE gel electrophoresis, and immunobloted with phosphor-GSK-3α/β antibody. For cell-based assay, cells were treated with different concentrations of CMEP for various time, analyzed, and cell lysates were used to conduct kinase assay as above.

2.3. Western blot analysis

CaP cells were treated with various concentrations of CMEP for various times. Equal amount of the cell lysates from the above-mentioned treatment were subjected to 4–20% SDS-PAGE gel electrophoresis, immunobloted with AKT [pS473], phospho-AKT (Thr308), p44/42 MAP kinase, PDK1, PI3K (Cell Signaling), AKT1 (Santa Cruz), AKT2, and AKT3 (Upstate), respectively. Blots were then incubated with horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence reagents subsequently (Amersham Pharmacia Biotech), followed by exposure to X-ray film (Kodak, Rochester, NY). Relative protein levels were quantified with the use of UN-SCAN-IT software (Silk Scientific Corp., Orem, Utah) on scanned films through digitization if necessary. Statistical analyses were carried out by student's *t*-test. Data in figures are expressed as means ± S.D. *p*-Values more than 0.05 were considered as not significant.

2.4. Cell growth inhibition assay

Cells were seeded in 96-well-plate and CMEP was prepared at three-fold dilutions in medium and incubated for various times. Cell viability was determined by cell counting kit-8 (Dojindo Inc., Gaithersburg, MD). The rationale of this kit is similar to that of the MTT assay, but with a novel tetrazolium salt that produces a water-soluble formazan dye upon bio-reduction [53].

2.5. Detection of apoptosis by flow cytometry

Cells undergoing apoptosis were detected by flow cytometry using a FACScan® (Becton Dickinson) with 488-nm laser line and analyzed using Cell Quest software. Phosphatidylserine exposed on the outside of the cells was determined by TACS™ Annexin V-FITC kit (Gaithersburg, MD). Briefly, cells were washed with cold PBS, pelleted and resuspended in 100 μl Annexin V-FITC diluted 1:100 in binding buffer (10 mM Hepes, 100 mM NaCl, 10 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$) containing propidium iodide (1:10). Cells were incubated for 10–15 min on ice, and then additional 400 μl binding buffer was added before FACScan® analysis.

2.6. In vivo inhibition of phospho-p70S6K and up-regulation of TSC2

Human CL-1 CaP tumor-bearing mice were from the untreated group of mice in the antitumor efficacy testing of other anticancer drugs (*e.g.*, Taxol). The animals selected all had one tumor in the dorsal flank on one side of the mice, with tumor volume about 2000 mm^3. CMEP was administered with i.p. or i.v. injection, at the dosage of 10 or 20 mg/kg per injection in 100 μl of 0.9% NaCl, one injection or three injections within 24 h. After the treatment, mice were sacrificed, and about 0.25 mm^3 of tumor tissue was used to extract protein with 1 ml lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% (v/v) Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, and 2 μg/ml aprotinin) in a Dounce homogenizer on ice, with 30 strokes, and followed by centrifugation at 4 °C to collect supernatants as protein samples. Equal amount of protein samples were then subjected to Western blot analysis, using TSC2, phospho (Thr389)-P70S6K, and GAPDH specific antibodies (Cell Signaling, Beverly, MA), respectively. All experiments were carried out following the guidelines of NIH and Institutional Animal Care and Ethics Committee.

3. Results

3.1. Basal AKT expression and activation levels in CaP cell lines

To understand how AKT is expressed in CaP cells, with Western blot analysis using specific antibodies, we characterized the expression levels of AKT1, AKT2, AKT3, phospho-Ser473 AKT, and phospho-Thr308 AKT in four CaP cell lines: DU-145, LNCaP, PC-3, and CL-1, an androgen-deprived subclone of LNCaP. As shown in Fig. 2A, PC-3 cells express the highest levels of AKT1, AKT2, and AKT3; DU-145 cells express moderate levels of AKT1, AKT2, and AKT3; whereas CL-1 and LNCaP cells express lower-levels of these three isoforms of AKT than those of the other two cell lines. On the other hand, DU-145 cells do not express either phospho-Ser473 AKT or phospho-Thr308 AKT, while CL-1 and LNCaP cells express higher-levels of these two than PC-3 cells. Interestingly, phospho-Ser473 AKT and phospho-Thr308 AKT show the same expression pattern, that is, the expression level of phospho-Ser473 AKT in CL-1 and LNCaP cells is higher than that in PC-3 cells while the expression level of phospho-Thr308 AKT in CL-1 and LNCaP cells is also higher than that in PC-3 cells. These results suggest that even though the expression

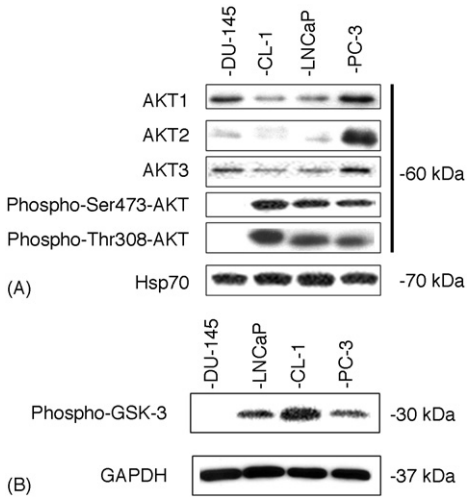

Fig. 2 – (A) Basal levels of AKT1, AKT2, AKT3, phospho-Ser473 AKT, and phospho-Thr308 AKT in four prostate cancer cell lines. Forty micrograms cell lysates from each cell line, which was reaching 90% growth confluence, were subject to 4–20% gradient Tris–glycine gel electrophoresis followed by immunoblot with AKT1, AKT2, AKT3, phospho-Ser473 AKT, and phospho-Thr308 AKT specific antibodies, respectively. Hsp70 reprobing showing equal loading of the total protein samples. (B) AKT kinase assay of four prostate cancer cell lines. One hundred micrograms cell lysates of DU-145, LNCaP, CL-1, and PC-3 were subjected to AKT kinase assay as described in Section 2. The AKT activity is shown by the phosphorylation of 30 kDa GSK-3 fusion protein, not the full-length 46 kDa GSK-3α/β protein (same for the following experiments). GAPDH reprobing showing equal loading of the protein samples. One representative from four different experiments showing similar results.

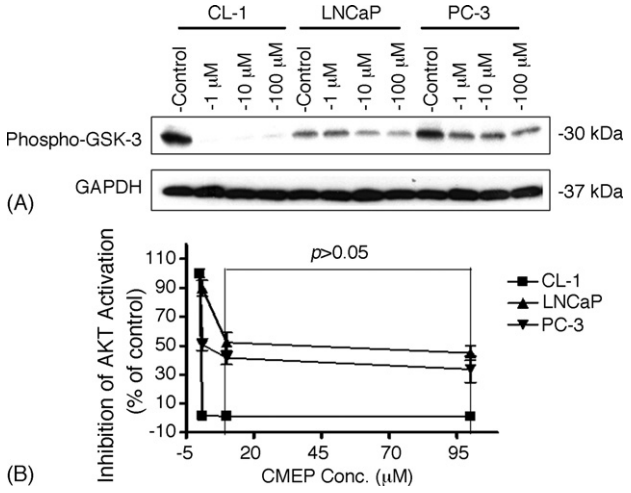

Fig. 3 – CMEP inhibits AKT activation in CaP cells with high-levels of AKT activation. Two hundred micrograms cell lysates of CL-1, LNCaP, and PC-3 were subjected to kinase assay as described in Section 2 with indicated concentration of CMEP. (A) Representative western blot is shown. GAPDH reprobing showing equal loading of the protein samples. (B) Quantification of the inhibition of AKT activation by scanning densitometry. Scanning data obtained from three independent experiments are expressed as a percentage of control (100%, maximal AKT activation, cells were treated with DMSO only) ±S.D.

levels of AKT isoforms vary in CaP cells, the expression levels of phospho-Ser473 AKT and phospho-Thr308 AKT are unique in each of the cancer cell lines, and are not necessarily correlated with the expression levels of AKT isoforms.

Next, to detect the basal AKT kinase activation levels in CaP cells, utilizing the lysates of four CaP cell lines DU-145, LNCaP, PC-3, and CL-1, we performed a cell-free based AKT kinase assay. We observed that these four CaP cells have distinct characteristics in their AKT activation. Among them, CL-1 cells have the highest levels of AKT activation, LNCaP and PC-3 cells have moderate levels of AKT activation but with LNCaP higher than PC-3 cells, while DU-145 cells have no AKT activation, as shown in Fig. 2B by phospho-GSK-3. Together with the AKT expression level data, this result demonstrates that the expression level phospho-Ser473 AKT correlates with AKT kinase activation in these four cell lines.

3.2. CMEP selectively inhibits activation of AKT in CaP cells

Using a bioinformatics-based approach, combined with AKT kinase assay, we identified a selective AKT small molecule inhibitor, 9-chloro-2-methylellipticinium acetate (CMEP). To reveal the relationship between the AKT inhibitory effect of CMEP and AKT activation in prostate cancer cells, we immunoprecipitated AKT from CL-1, LNCaP, and PC-3 cells, then conducted an AKT kinase assay in the presence of different concentrations of CMEP. As seen in Fig. 3A, among these three cell lines, CL-1, which has the highest level of AKT activation, was the most susceptible one, as the p30 band of phospho-GSK-3 disappeared even at as low as 1 μM of CMEP

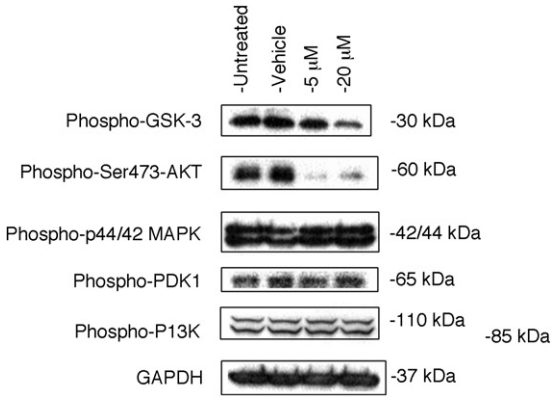

Fig. 4 – CMEP inhibits only AKT, does not inhibit phospho-PDK1, P42/p44 MAPK, or PI3K. 3×10^5 PC-3 cells were treated with desired concentrations of CMEP for 48 h, cell lysates were harvested and subjected to AKT kinase assay and Western blot analysis as described in Section 2. GAPDH reprobing was used to show equal loading of the total protein samples. One representative from three independent experiments showing similar results.

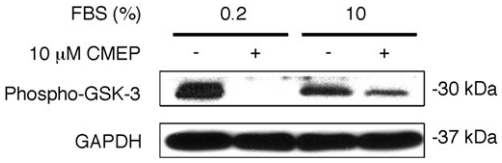

Fig. 5 – CMEP inhibits serum-starvation-induced activation of AKT. 1×10^6 PC-3 cells in different concentration of FBS was cultured for 24 h, then treated with CMEP for 30 min, 200 μg harvested lysates were subjected to AKT kinase assay as described in Section 2. GAPDH reprobing showing equal loading of the protein samples. One representative from three different experiments showing similar results.

treatment. In LNCaP cells, the p30 band became weak at 10 μM of CMEP treatment whereas as low as 1 μM of CMEP treatment started to inhibit AKT activation in PC-3 cells, indicating that CMEP is more potent in inhibiting AKT activation in PC-3 cells than in LNCaP cells. Also, in each of these two cell lines, the AKT inhibitory effect of CMEP seemed to reach a plateau at 10 μM of CMEP treatment, since there was no significant difference between 10 and 100 μM of CMEP treatment ($p > 0.05$, Fig. 3B). Compared to CL-1 cells, even though CMEP clearly inhibits AKT activation in LNCaP and PC-3 cells, this effect is not as potent as in CL-1 cells. Both LNCaP and PC-3 cells have high-levels of AKT activation, but lower than that of CL-1 cells. These results suggest that CMEP preferentially inhibits AKT activation in cancer cells with high-levels of AKT activation.

As a representative, we then did a cell-based AKT kinase assay in PC-3 cells treated with 5 and 20 μM of CMEP to see how CMEP acts at cellular level. We observed that treated with CMEP for 48 h, CMEP inhibited the AKT kinase activation and phospho-Ser473 AKT, this result indicates that CMEP penetrates cell membrane and acts intra-cellularlly to inhibit AKT, as shown in Fig. 4.

Furthermore, to clarify if CMEP inhibits other kinases, we probed phospho-p42/44 MAPK, phospho-PDK1, and phospho-PI3K in the same cell lysates using Western blot analysis with specific antibodies against these three kinases. We found that CMEP did not inhibit phospho-p42/44 MAPK, phospho-PDK1 kinase, or phospho-PI3K as also demonstrated in Fig. 4.

Therefore, CMEP inhibits AKT activation in cancer cells with high-levels of AKT activation. CMEP inhibits only AKT, does not affect PI3K, PDK1, or MAPK.

3.3. *CMEP inhibits serum-starvation-induced activation of AKT*

As a cell survival factor, AKT can be activated under a variety of stimuli including serum-starvation. To investigate whether CMEP inhibits AKT activation under this condition, we tested the ability of CMEP in blocking serum-starvation-induced AKT activation also in PC-3 cells. As shown in Fig. 5, PC-3 cells were cultured in the medium containing different concentrations of FBS for 24 h, then cells were treated with 10 μM CMEP for 30 min, and cell lysates were subjected to AKT kinase assay. We observed that AKT activation was higher in medium with 0.2% FBS than that with 10% FBS, and the higher-level of AKT kinase activation under serum-starvation condition seemed to be more sensitive to the inhibitory effect of CMEP than the lower-level of AKT kinase activation in medium with 10% FBS. This is consistent with the AKT-inhibition effect by curcumin observed in CaP cells [44], indicating that CMEP inhibits inducible AKT activation.

3.4. *CMEP selectively inhibits growth in CaP cells with high-levels of AKT activation*

AKT transmits the survival/over-growth signal in cancer cells. To investigate whether CMEP blocks this signaling process by inhibiting growth of cancer cells with high-levels of AKT activation, we performed a serial of growth assays to test the inhibitory effect of CMEP on CaP cells. First, we treated four CaP cell lines CL-1, DU-145, LNCaP, and PC-3 with a serial doses of CMEP with regular culture medium supplemented with 10% FBS for different times, then determined the cell viability with Cell Counting Kit-8. As shown in Fig. 6, CMEP inhibits cell proliferation of CL-1, DU-145, LNCaP, and PC-3 cells in a dose- and time-dependent manner. We observed that the growth-inhibitory effect of CMEP on these cell lines started at as early as at 24 h of treatment. If compared to the cells survived, we found that among these four cell lines, CL-1 was the most sensitive cell line to CMEP-induced growth inhibition (Fig. 6B); LNCaP was next (Fig. 6C); PC-3 was moderately sensitive (Fig. 6D); whereas DU-145 was relatively resistant (Fig. 6A). Interestingly, we observed that at lower dose (<2 μM), CMEP reached its highest level of growth inhibition against CL-1 cells between 48 and 72 h of treatment, and after that time period, this inhibitory effect was lost, and CL-1 cells grew back to its starting number. Combined with the basal AKT activation levels of these four CaP cell lines as exhibited in Fig. 2A and B, this result illustrates that CMEP preferentially inhibits growth of CaP cells with high-levels of AKT activation.

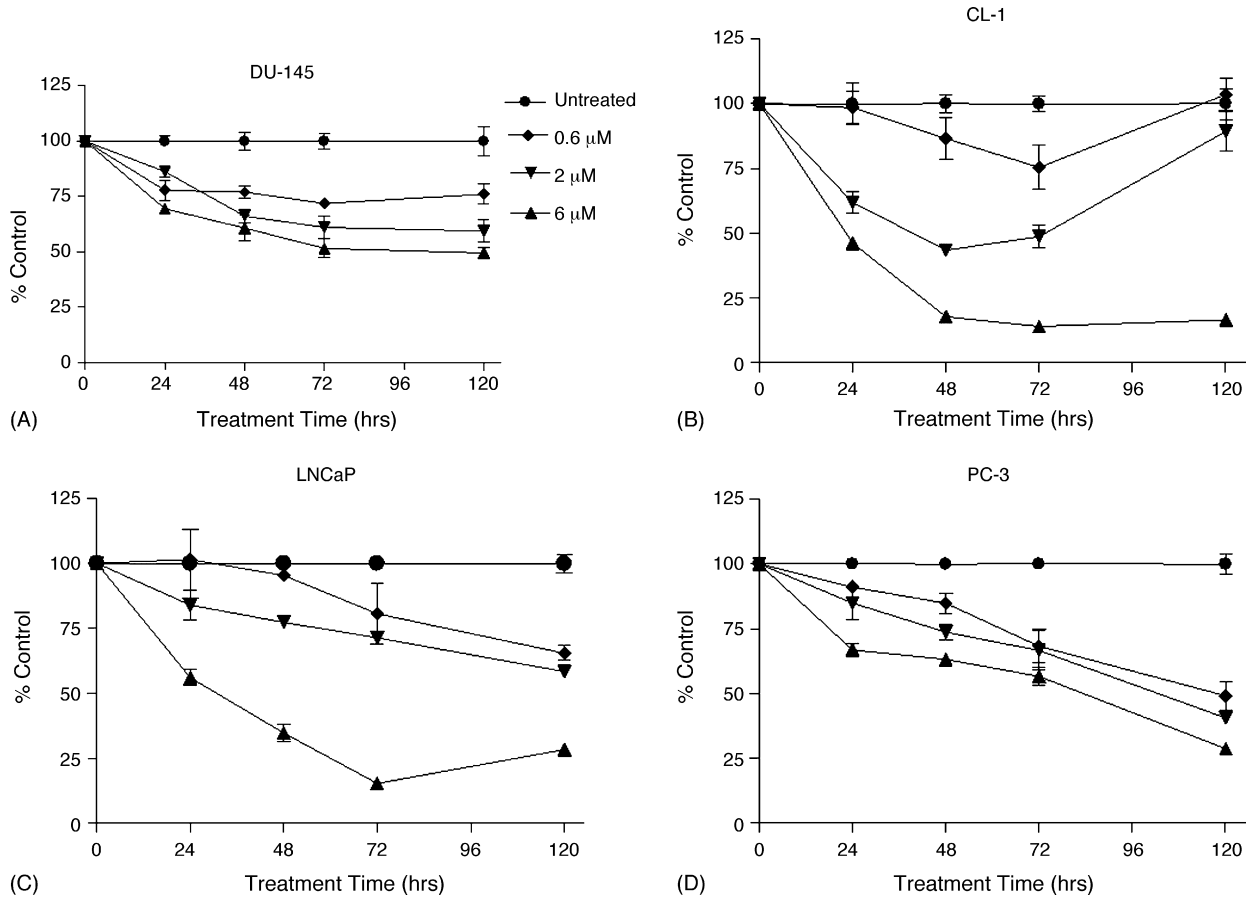

Fig. 6 – Time- and dose-dependent growth inhibition of prostate cancer cells by CMEP. 5×10^4 DU-145 (A), CL-1 (B), LNCaP (C), and PC-3 (D) prostate cancer cells cultured in the media containing 10% FBS were treated with different concentrations of CMEP for different times, then cell viability was tested with Cell-Counting Kit-8. Data shown are the values of mean and S.D. from four different experiments.

3.5. *CMEP induces apoptosis in CaP cells with high-levels of AKT activation*

Apoptosis, or programmed cell death, is a very important form of cell death. Cancer cells with high-levels of AKT activation are so sensitive to the growth inhibitory effect of CMEP, it would be of great significance to see whether CMEP induces apoptosis in these cells. To test the apoptosis-inducing effect of CMEP on CaP cells, we first treated DU-145 (no AKT activation), CL-1 (higher-levels of AKT activation), LNCaP (high-levels of AKT activation), and PC-3 (high-levels of AKT activation) cells with 10 μM of CMEP for 48 h, then we stained the cells with both Annexin V-FITC and propidium iodide, followed by flow cytometry analysis. We found that about 80% of CL-1 cells, 70% of LNCaP cells, and 55% of PC-3 cells were undergoing apoptosis, but this rate was only about 35% in DU-145 cells, as seen in Fig. 7. This result indicates that CMEP preferentially induces apoptosis in CaP cells with high-levels of AKT activation.

3.6. *CMEP inhibits phospho-p70S6K and up-regulates TSC2 in vivo*

As two important downstream targets of AKT, accumulating evidence has shown that TSC1/TSC2 controls cell size and p70S6K controls protein synthesis, up-regulated TSC1/TSC2 decreases cell size, while up-regulated phospho-p70S6K increases protein synthesis [23,27,28], implicating that increased expression of TSC2 and decreased expression of phosphor-p70S6K result in inhibiting tumor growth. To correlate the regulation of TSC2 and phospho-S6K with the modulation of phospho-Ser473 AKT by the treatment of CMEP in CL-1 CaP xenograft model, one of the most aggressive tumor models of CaP [52], to shed light that CMEP may inhibit tumor growth, we probed the protein samples from the tumor tissue from the mice treated with CMEP with Western blot analysis by using phospho-Ser473 AKT, TSC2, and phospho-p70S6K specific antibodies, respectively. As can be seen in Fig. 8A, given 10 or 20 mg/kg of CMEP per injection, i.p., three injections per mouse in mice-bearing big tumors (2000 mm^3) within 24 h, phospho-Ser473 AKT was inhibited about 10–40% in different mice while TSC2 was stimulated and phospho-p70S6K was inhibited in the tumor tissue. Similarly, given 10 or 20 mg/kg of CMEP per injection, i.v., one injection or three injections per mouse in the other group of mice-bearing similar size of tumors in 24 h, phospho-Ser473 AKT was also inhibited about 10–35% in different mice while TSC2 was stimulated and phospho-p70S6K was inhibited in the tumor tissue. It appeared that 20 mg/kg of CMEP per injection, three injections within 24 h had

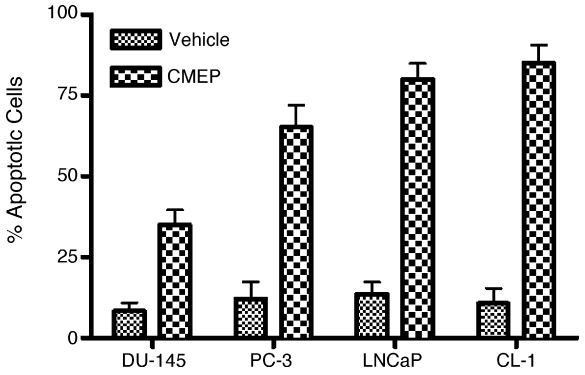

Fig. 7 – CMEP induces apoptosis in prostate cancer cells with high-levels of AKT activation. 2 × 10^6 DU-145, CL-1, LNCaP, and PC-3 cells cultured in the media containing 10% FBS were treated with 10 μM CMEP for 48 h, cells were then subjected to flow cytometry analysis following Annexin V–propidium iodide double staining as described in Section 2. Data shown are the values of mean and S.D. from three independent experiments.

better effect than the other treatments, as exhibited in Fig. 8B. In both cases, CMEP treatment did not affect AKT. In addition, even though there were variations between each individual mouse, the alterations of phospho-Ser473 AKT, TSC2, and phospho-p70S6K were very clear, compared to the mouse treated with only 0.9% NaCl. These data suggest that CMEP may inhibit tumor growth, at least, by up-regulation of TSC2 and down-regulation of phospho-p70S6K through inhibiting, at least, phospho-Ser473 AKT.

4. Discussion

AKT, PTEN, p53, and AR all contribute to the control of CaP cell growth and metastasis. Four CaP cell lines, CL-1, DU-145, LNCaP, and PC-3, which have been used to study the anticancer activity of CMEP, have distinct characteristics regarding their basal levels of AKT activation, PTEN expression status, p53 expression status, and androgen-dependency, as summarized in Table 1.

CMEP has a similar structure to that of 9-hydroxyellipticine, which has been reported to have unique anticancer activity [54] of inhibiting mutant p53 protein phosphorylation, thereby restoring the function of wild-type p53 [55]. Both CL-1 and LNCaP cells have wild-type of p53, PC-3 and DU-145 cells have either null or mutant p53; CL-1 and LNCaP cells are relatively

Table 1 – Characteristics of four CaP cell lines[a]

Cell Lines	AKT activation	PTEN	p53	Androgen-dependency
CL-1	+++	mt	wt/wt	–
DU-145	–	+	mt/mt	–
LNCaP	++	mt	wt/wt	+
PC-3	+	–	Null	–

[a] Also based on [2–4].

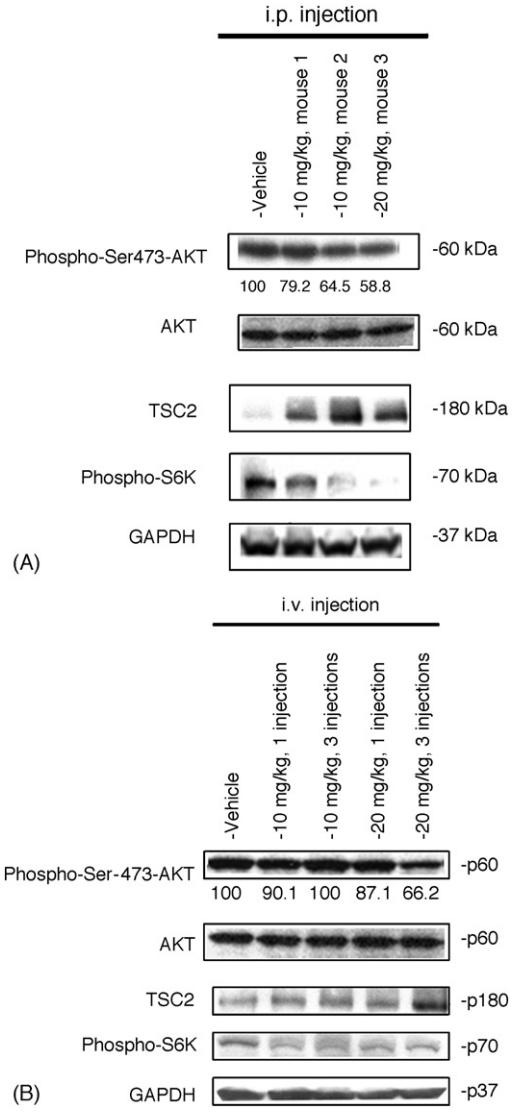

Fig. 8 – (A) Four CL-1 CaP tumor-bearing mice were given desired dosage of CMEP or 0.9% NaCl with i.p., three injections in 24 h. (B) Five CL-1 CaP tumor-bearing mice were given 0.9% NaCl or CMEP with desired dose schedule, i.v. injection in 24 h. Protein samples from the tumor tissue were subjected to Western blot analysis using TSC2 and phospho (Thr389)-S6K antibodies, respectively. GAPDH reprobing showing equal loading of the protein samples.

more sensitive than PC-3 and DU-145 cells to CMEP-induced growth inhibition while PC-3 cells are relatively more sensitive than DU-145 cells to CMEP-induced growth inhibition, indicating that the anticancer effect of CMEP still requires functional p53, and CMEP may restore the function of wild-type p53 in PC-3 cells.

CL-1 cell line, which has a higher-level of AKT activation than its parental cell line, LNCaP, grows without androgen. On the other hand, DU-145 also grows without androgen, but there is no or undetectable level of AKT activation in DU-145. One possible interpretation is that even with mutant p53, functional PTEN expression in DU-145 [32] might be sufficient

to antagonize AKT activation from androgen deprivation; in LNCaP cells [56], wild-type p53 does not have this ability to antagonize this activation with mutant PTEN. This suggests that the interaction between AKT and PTEN is probably a more upstream event of p53.

CL-1 cells have higher-levels of AKT activation than that of LNCaP and PC-3 cells (Fig. 2B), AKT activation in CL-1 cells is more sensitive to the inhibitory effect of CMEP than that of LNCaP and PC-3 cells (Fig. 3A). Moreover, in PC-3 cells CME inhibits the higher-level of AKT activation from serum-starvation condition much more potent than that from normal serum condition (10%), which means serum-starvation resulting in the cells becoming more susceptible to the inhibitory effect of CMEP by activating more AKT. These observations suggest that CMEP preferentially inhibits high-levels of AKT activation in CaP cells, i.e., the higher-level of AKT activation, the more susceptible the cells the inhibitory effect of CMEP.

The inhibitory effect of CMEP on AKT activation varies in three CaP cell lines (Fig. 3A), the dose-dependent inhibitory effect of CMEP is clearer in PC-3 cells than that in LNCaP cells in the dose range of less than 10 μM; no significant differences between 10 and 100 μM ($p > 0.05$, Fig. 3B), indicating the inhibitory effect reached a plateau started from 10 μM of CMEP treatment at current condition. CL-1 is still the most susceptible one; the clear dose-dependency in CL-1 is in the dose range of less than 1 μM. On possible explanation for the different potency and dose-dependency of CMEP is that besides the level of AKT activation, CMEP may preferentially inhibit AKT activation in cells growing without androgen, implicating that CMEP may be an effective reagent for the treatment of hormone-refractor CaP. From Table 1, we can see that the growth of CL-1 and PC-3 is androgen-independent while LNCaP is androgen-dependent.

PTEN is a negative regulator of AKT [7–11,32,33]. The experiment results in this report show that CMEP preferentially inhibits growth and induces apoptosis in CaP cells with high-levels of AKT activation, such as CL-1, LNCaP, and PC-3, but all these three cell lines do not express functional PTEN. On the other hand, DU-145 cells, which express functional PTEN and are tumorigenic, but they are relatively resistant to the growth inhibitory and apoptosis-inducing effects of CMEP, one explanation is that DU-145 cells do not have AKT activation, therefore lack the specific target for CMEP. So it is possible that the anticancer effect of CMEP does not require functional PTEN, but requires AKT activation.

CMEP inhibits AKT, but does not inhibit PI3K, PDK1, or p44/42 MAPK. Since the kinases detected in this work are very limited, even though these three kinases are the major upstream or downstream targets of AKT, CMEP may still affect other kinases.

Also, at current stage of development, the selectivity of CMEP in terms of growth inhibition and apoptosis induction is not so optimal by modulating other survival signal transduction pathways instead of AKT, These are probably the reasons that CMEP has some limited effect on DU-145 cells which do not have AKT activation, compared to CL-1, PC-3, and LNCaP which all have high-levels of AKT activation. Therefore, more kinases will be needed to test CMEP and further structure-activity relationship (SAR) will be required to improve the potency and selectivity of this compound.

CL-1, an androgen-deprived subclone of LNCaP [33,52], is more sensitive than LNCaP cells to CMEP-induced growth inhibition. Both CL-1 and LNCaP do not express functional PTEN. The only difference between CL-1 and LNCaP is their androgen-dependency, CL-1 can grow without androgen. Using this pair of cell lines, it would be also interesting to address and clarify these issues: (1) whether or not AR-signaling is dependent on the activation of AKT, (2) whether or not AR-signaling is dependent on the function of PTEN, and (3) how AKT and PTEN cooperate to contribute to AR-signaling.

Two different assays were used in this report: one is to evaluate the AKT activation inhibitory effect of CMEP using a certain amount of CaP cell lysates (*in vitro* cell-free based AKT kinase assay), such as shown in Fig. 3, the other is to evaluate the growth inhibitory effect of CMEP over a certain period of time (cell-based assay), such as shown in Fig. 6. This may in part explain 1 μM of CMEP strongly inhibited AKT activation in CL-1 cells in Fig. 3 but not so potent in inhibiting CL-1 growth in Fig. 6B. As a matter of fact, at lower dose (≤2 μM), CMEP reached its highest level of growth inhibition against CL-1 cells between 48 and 72 h of treatment, after that this inhibitory effect was lost, and CL-1 cells grew back to its starting number. This is probably because that CL-1 is a fast-growing cell line when compared with its parental cell line, LNCaP [33], and the other CaP cell lines used in this work, a rapid self-renewal may occur at lower dose of CMEP treatment after certain period of time. Compared to LNCaP, CL-1 not only has a higher-level of AKT activation, in a separate effort to develop Bcl-2 small molecule inhibitor anticancer drugs, we found that CL-1 has a higher-level of Bcl-2 expression (Zhang, et al., unpublished data), Bcl-2 is also an important survival factor. These data suggest that androgen deprivation result in dramatic activation of survival signals in CL-1 cells. PC-3 cells grow faster than LNCaP cells, this may explain that PC-3 cells are more sensitive than LNCaP cells to the inhibitory effect of CMEP in *in vitro* cell-free based kinase assay (Fig. 3) while PC-3 cells are relatively more resistant than LNCaP cells to the growth inhibitory effect of CMEP (Fig. 6D).

TSC1–TSC2 negatively controls cell size, also regulates the downstream phospho-4E-BP1 and phospho-p70S6K, which positively control protein synthesis to provide cell mass [23,27–30]. Increased cell size and cell mass contribute to tumor growth due to down-regulation of TSC1-TSC2 and up-regulation of phospho-4E-BP1 and phospho-p70S6K. In this report, we treated limited number of tumor-bearing mice with CMEP for only a limited period of time, still we have observed that CMEP inhibits phospho-Ser473 AKT within only 24 h of treatment, although not so dramatic with only a maximal level of about 40% of inhibition, this inhibition was enough to trigger the up-regulation of TSC2 and down-regulation of phospho-p70S6K, so we predict from this pilot experiment that the tumor growth will be greatly inhibited with a enough time of treatment by CMEP, at least, via up-regulation of TSC2 and down-regulation of phospho-p70S6K through inhibiting at least, phospho-Ser473 AKT.

CMEP is an AKT activation inhibitor; it does not modulate the expression of AKT, as shown in this work (Fig. 8A and B). It is understood that CMEP has no or minimal effect on cells without AKT by siRNA knock down because of lacking in appropriate molecular target.

In conclusion, by selectively blocking AKT activation, CMEP preferentially inhibits growth and induces apoptosis in CaP cells which have high-levels of AKT activation, lack PTEN or harbors PTEN mutation. CMEP may inhibit tumor growth in CaP xenograft mouse model, at least, through stimulating TSC2 and inhibiting phospho-p70S6K.

The discovery of CMEP has, at least, two outcomes. First, using CMEP as a powerful research tool, biological and biochemical mechanism studies will further our understanding of the roles of AKT in regulating cell growth and apoptosis, and its interactions with other targets in cell survival-signaling pathways. Second, this study will benefit the efforts toward designing and discovering more potent small molecule inhibitors of AKT as potential therapy for the treatment of CaP, especially hormone-refractory CaP, in which AKT is highly activated and for which traditional therapy has failed.

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