Anti-growth Effect of 1,25-Dihydroxyvitamin D_3-3-bromoacetate Alone or in Combination with 5-Amino-imidazole-4-carboxamide-1-β-4-ribofuranoside in Pancreatic Cancer Cells

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Abstract. *1,25-Dihydroxyvitamin D_3-3-bromoacetate ($1,25(OH)_2D_3$-3-BE) is a vitamin D receptor-alkylating derivative of $1,25(OH)_2D_3$. The strong dose-dependent anti-proliferative and apoptotic effects of this compound in androgen-sensitive and androgen-insensitive prostate cancer cells have been reported. In this communication, it is reported that $1,25(OH)_2D_3$-3-BE strongly inhibits the growth of several pancreatic cancer cell lines. This effect is further accentuated by combination with 5-amino-imidazole-4-carboxamide-1-β-4-ribofuranoside (AICAR), an activator of AMP-activated protein kinase (AMPK)/acetyl-Co-enzyme A carboxylase (ACC) phosphorylation pathways and an inhibitor of Akt phophorylation. It was observed that the anti-growth property of $1,25(OH)_2D_3$-3-BE, either alone or in combination with AICAR resulted in the inhibition of Akt phosphorylation in BxPC-3 cells. In conclusion, $1,25(OH)_2D_3$-3-BE displays a strong therapeutic potential, alone and in combination with AICAR, in pancreatic cancer.*

Pancreatic cancer (PAC) is the fourth most common cause of cancer related deaths in the United States, totaling approximately 32,000 fatalities per year (1). The rate of incidence of PAC is roughly the same as the rate of mortality, and five-year survival rate is less than 1%. This poor prognosis can be attributed to several factors, including propensity of the tumor to metastasize even when it is small, late detection at an advanced and often metastasized state, and intrinsic resistance to therapies with radiation and cytotoxic agents such as 5-fluorouracil, gemcitabine, either alone, or in combination (2). Certain natural products, *e.g.* genistein, have also shown limited effects in pancreatic cancer cells (3, 4).

1,25-Dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), an essential nutrient for skeletal health, has strong antiproliferative effect in various cancer cells (5). However, results of several clinical studies have shown that the beneficial effect of $1,25(OH)_2D_3$ in therapeutic doses is exacerbated by its strong calcemic toxicity, leading to a search for analogs of $1,25(OH)_2D_3$ with antiproliferative activity and reduced toxicity (5, 6). In an alternative approach, combinations of $1,25(OH)_2D_3$ with standard chemotherapeutic agents have shown promise in mitigating toxicity related to $1,25(OH)_2D_3$ (7-12). Additionally, pre-treatment with $1,25(OH)_2D_3$ or one of its analogs has been shown to increase sensitivity towards radiation in certain breast and prostate cancer cells (13-15).

An alkylating derivative of $1,25(OH)_2D_3$ (1,25-dihydroxyvitamin D_3-3-bromoacetate, $1,25(OH)_2D_3$-3-BE) has been developed which covalently labels the hormone-binding pocket of nuclear vitamin D receptor (VDR) (16, 17). It has been reported previously that $1,25(OH)_2D_3$-3-BE, as well as 25-hydroxyvitamin D3-3-boromoacetate (25-OH-D_3-3-BE), a prototype of $1,25(OH)_2D_3$-3-BE without the 1-hydroxyl group, are considerably stronger antiproliferative agents than $1,25(OH)_2D_3$ in several prostate cancer cells (18-21). In this communication, it is demonstrated that $1,25(OH)_2D_3$-3-BE displays a strong antiproliferative property in PAC cells as well, and that this activity is strongly enhanced by co-dosing with 5-amino-imidazole-4-carboxamide-1-β-4-ribofuranoside (AICAR), an activator of AMP-activated protein kinase (AMPK)/acetyl-Co-enzyme A carboxylase (ACC) phosphorylation pathways (22).

Materials and Methods

Cellular assays. Standard ^{3}H-thymidine incorporation (in HS766 cells) and MTT assays (in ASPC-1 cells), and growth assays (in BxPC-3, HS766 and MiaPaca cells) were employed in order to

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evaluate the cellular activities of $1,25(OH)_2D_3$ (a gift from Dr. Milan Uskokovic, Hoffman La-Roche, Inc., Nutley, NJ, USA), $1,25(OH)_2D_3$-3-BE (23), AICAR (Toronto Research Chemicals, Ontario, Canada) and a combination of $1,25(OH)2D_3$-3-BE and AICAR. Cell lines were purchased from ATCC (Manassas, VA, USA) and cultured and propagated according to manufacturer's instructions.

In general, cells were incubated with various doses (as denoted in figure legends) of $1,25(OH)_2D_3$-3-BE, $1,25(OH)_2D_3$ or ethanol (vehicle) in media containing 5% fetal bovine serum for 16 h followed by ^{3}H-thymidine-incorporation or MTT assays. In the growth assay BxPC-3, HS766 and MiaPaca cells were treated with various agents $1,25(OH)_2D_3$-3-BE, $1,25(OH)_2D_3$ or AICAR, either individually or in combination on days 1, 3 and 5. On the seventh day cells were trypsinized and counted using a hemocytometer. Cells were dosed with $1,25(OH)_2D_3$ and $1,25(OH)_2D_3$-3-BE, dissolved in 0.1% v/v EtOH, or AICAR, dissolved in 0.1% v/v DMSO. For combination studies, appropriate solutions were mixed in media to maihtain concentrations of EtOH and DMSO at 0.1% v/v or less. All assays were repeated six times and statistical analysis was performed by Student's *t*-test.

Western blot analysis. Lysates were made from treated cells in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 7.5, 50 mM NaF, 1 mM sodium vanadate and protease inhibitors; Sigma-Aldrich, Milwaukee, WI, USA), and protein homogenates (50 μg) were run either on a 4-15% SDS polyacrylamide gel (Biorad, Hercules, CA, USA) or 4-12% MES NuPAGE gels (Invitrogen, Carlsbad, CA, USA) and transferred onto a polyvinylidene fluoride (PVDF) membrane. Analyses were carried out with primary antibodies for P-AMPK (Thr 172), AMPK, ACC and P-ACC (Ser 79) (Upstate Biotechnology, Charlottesville, VA, USA) at a dilution of 1:1000. Akt and P-Akt (Ser 473) (Cell Signaling, Danvers, MA, USA) were used at a dilution of 1:3000 and 1:2000 respectively. Dilutions for p21 (Santa Cruz, Biotehnology, Santa Cruz, CA, USA and p53 antibodies (Abcam Inc, Cambridge, MA, USA) were 1:500 and 1:1000 respectively. A secondary antibody conjugated to horseradish peroxidase (GE Healthcare, UK) was used at 1:5000 dilution. Signals were detected by chemiluminescence solution (Pierce, Rockford, IL, USA or Perkin Elmer, Boston, MA, USA) and autoradiography.

Results

$1,25(OH)_2D_3$-3-BE strongly inhibits the growth of ASPC-1, BxPC-3, HS766 and MiaPaca cells. Results of ^{3}H-thymidine and MTT assays are shown in Figures 1A and B. $1,25(OH)_2D_3$ at 10^{-8}-10^{-6}M had negligible effect in the growth of these cells, while there was approximately 60 and 20% reduction in growth with 10^{-7}M of $1,25(OH)_2D_3$-3-BE, in ASPC-1 and HS766 cells respectively. With 10^{-6}M of $1,25(OH)_2D_3$-3-BE inhibition of growth was approximately 75% and 60% in ASPC-1 and HS766 cells, respectively.

In the growth assay with BxPC-3 cells, growth inhibition by 10^{-8}-10^{-6}M of $1,25(OH)_2D_3$ were approximately 0%, 20% and 40% respectively, while the same doses of $1,25(OH)_2D_3$-3-BE caused approximately 10%, 95% and 100% growth inhibition respectively (Figure 1C). In MiaPaCa cells, 10^{-7}M, 5×10^{-7}M and 10^{-6}M of $1,25(OH)_2D_3$-3-BE caused approximately 65%, 90% and 95% growth inhibition respectively, while the same doses of $1,25(OH)_2D_3$ resulted in approximately 0%, 5% and 10% inhibition of growth respectively (Figure 1D).

A combination of $1,25(OH)_2D_3$-3-BE and AICAR strongly and synergistically inhibits the growth of BxPC-3 cells. It was observed that 10^{-5}M of AICAR had no significant effect on the growth of BxPC3 and HS766 cells, but 10^{-4}M and 10^{-3}M of AICAR strongly suppressed their growth, indicating low-efficacy of AICAR in inhibiting the growth of these cells (Figure 2A). However, when cells were co-dosed with $1,25(OH)_2D_3$-3-BE, a strong growth-inhibition of BxPC-3 cells was observed (Figure 2B). For example, 3×10^{-5}M of AICAR had no effect on cellular growth, while 3×10^{-7}M of $1,25(OH)_2D_3$-3-BE inhibited the growth by approximately 60%. When $1,25(OH)_2D_3$-3-BE and AICAR were combined (at the same doses as in individual dosing) growth inhibition increased to approximately 85%.

Evaluation of cell signaling pathways in BxPC-3 cells. AMPK and ACC-phosphorylation: As shown in Figure 3A, both phopho-AMPK and phopho-ACC are up-regulated by AICAR in a dose-dependent manner.

AMPK phosphorylation in BxPC-3 cells was also evaluated when they were treated with AICAR (10^{-4}M) and $1,25(OH)_2D_3$-3-BE (3×10^{-7}M) either individually or in combination. Results in Figure 3B demonstrate that AICAR strongly activated the level of phospho-AMPK, while $1,25(OH)_2D_3$-3-BE reduced its level significantly. The level of phospho-AMPK was partly restored by combining the two reagents.

Modulation of p21 and p53: Results of these assays are shown in Figure 4, left panel. The level of p21 was up-regulated by $1,25(OH)_2D_3$-3-BE, but not by AICAR, and combination of the two essentially reflects the level produced by $1,25(OH)_2D_3$-3-BE alone. In contrast, AICAR strongly up-regulated the level of p53, while $1,25(OH)_2D_3$-3-BE did not change the level (from control) significantly. The combination, however, showed the strongest signal.

A combination of AICAR and $1,25(OH)_2D_3$-3-BE strongly inhibited Akt-phosphorylation. Results of the Western Blot analysis are shown in Figure 4, right panel. AICAR (6×10^{-5}M) strongly inhibited Akt phosphorylation, while $1,25(OH)_2D_3$-3-BE (10^{-7}M) had a considerably weaker effect. However, when the cells were dosed with a combination of the two reagents Akt phosphorylation was almost completely eliminated.

Discussion

It has been reported that $1,25(OH)_2D_3$-3-BE and 25-OH-D_3-3-BE, alkylating derivatives of $1,25(OH)_2D_3$ and 25-OH-D_3 respectively are considerably stronger antiproliferative agents than $1,25(OH)_2D_3$ in several prostate cancer cells (18-21). The

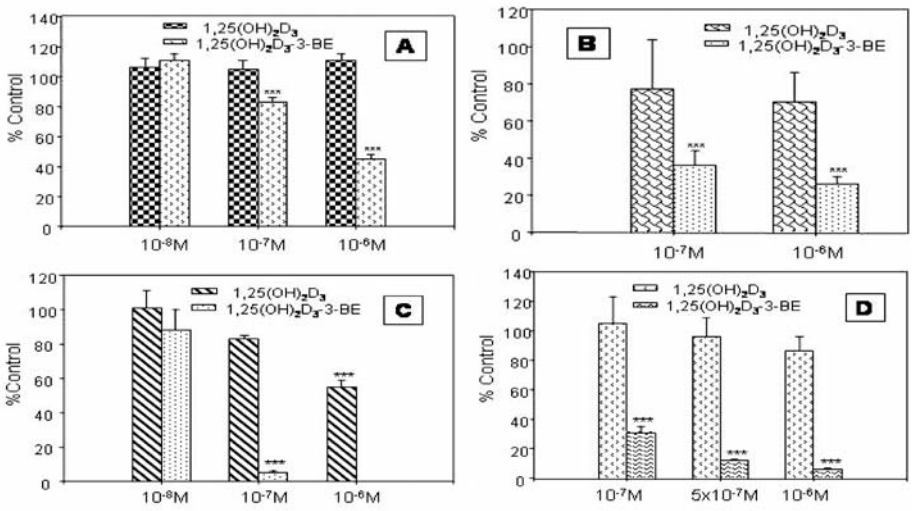

Figure 1. *A: ^{3}H-Thymidine incorporation assay of HS766 cells treated with various doses of 1,25(OH)$_2$D$_3$, 1,25(OH)$_2$D$_3$-3-BE or ethanol (control) for 16 h followed by standard ^{3}H-thymidine incorporation assay. B: MTT assay of ASPC-1 cells dosed with 1,25(OH)$_2$D$_3$, 1,25(OH)$_2$D$_3$-3-BE or ethanol (control) for 16 h. C, D: Growth assay of BxPC-3 and MiaPaca cells respectively, where cells were treated with various doses of 1,25(OH)$_2$D$_3$, 1,25(OH)$_2$D$_3$-3-BE or ethanol (control) on 1st, 3rd and 5th days. On the 7th day cells were trypsinized and counted in a hemocytometer. Results represent six replicates. Statistical analysis was carried out by Student's t-test (***p<0.001).*

present study was carried out to determine the effect of 1,25(OH)$_2$D$_3$-3-BE in PAC cells.

First, we evaluated the growth-inhibitory property of 1,25(OH)$_2$D$_3$, 1,25(OH)$_2$D$_3$-3-BE and AICAR in various PAC cells by three assays, namely ^{3}H-thymidine assay, an MTT assay and a growth assay. In the ^{3}H-thymidine assay, incorporation of radioactive thymidine in the DNA of rapidly growing cells is measured. Therefore, for this assay a short treatment period (16 h) was employed with relatively higher doses (up to 10^{-6}M) to observe a fast and maximally observable activity. Similarly, in the MTT assay cell viability was measured after a relatively quick treatment and high doses. As shown in Figure 1A and B 1,25(OH)$_2$D$_3$-3-BE strongly inhibited the growth of ASPC-1 and HS766 cells, while equivalent amounts of 1,25(OH)$_2$D$_3$ showed negligible activity towards the growth of these cells.

In the growth assay, cells were treated for a longer period (7 days) with chronic dosing of reagents on the 1st, 3rd and 5th days followed by cell counting on the 7th day. This dosing regimen mimicked the chronic dosing of a clinical situation with a relatively lower dose of the reagents to be used. In BxPC-3 and MiaPaca cells, 1,25(OH)$_2$D$_3$-3-BE strongly inhibited the growth in a dose-dependent manner, while only 10^{-6}M of 1,25(OH)$_2$D$_3$ showed significantly lower activity compared with an equivalent dose of 1,25(OH)$_2$D$_3$-3-BE (Figures 1C and D). Overall, these results showed that 1,25(OH)$_2$D$_3$-3-BE, but not 1,25(OH)$_2$D$_3$, has a strong growth-inhibitory effect in PAC cells.

The well-known resistance of pancreatic cancer cells towards chemotherapy results from evasion of apoptosis/cell cycle inhibition, which can occur *via* multiple pathways. Therefore, it can be hypothesized that a combination of cytoxic agents that uses multiple pathways for inhibiting cell-growth may potentially be more effective than a single agent. AICAR is an activator of AMPK which phosphorylates and down-regulates a number of enzymes in the energy-metabolism pathway, such as ACC, fatty acid synthase, 3-hydroxy-3-methylglutaryl-CoA reductase, mammalian target of rapamycin (mTOR) *etc*. (24-32). Recently it was demonstrated by Rattan *et al*. that AICAR inhibits the growth of several cancer cell lines by activating cell cycle inhibitory proteins p21, p27, and p53 (22). It was also shown that AICAR activates AMPK and ACC phosphorylation, as well as mTOR in these cells, but inhibits Akt phosphorylation (22). These results suggested that the growth-inhibitory property of AICAR may be mediated by the inhibition of the PI3K-Akt pathway and activation of cell-cycle regulatory proteins. It was also demonstrated that AICAR is effective in a rat model of glioma (22).

It was our intention to evaluate whether combining AICAR with 1,25(OH)$_2$D$_3$-3-BE might enhance the growth-inhibitory activity of the latter in PAC cells. As shown in Figure 2A, AICAR is too low in potency to reduce the growth of BxPC-3 cells (Figure 2A). But when a low dose (3×10^{-5}M) of AICAR, with no cell-regulatory activity, was combined with 3×10^{-7}M of 1,25(OH)$_2$D$_3$-3-BE, cell-growth inhibition increased from 60% (with 1,25(OH)$_2$D$_3$-3-BE alone) to 85%. These results indicated that the growth-inhibitory property of 1,25(OH)$_2$D$_3$-3-BE is strongly accentuated by AICAR in BxPC-3 cells.

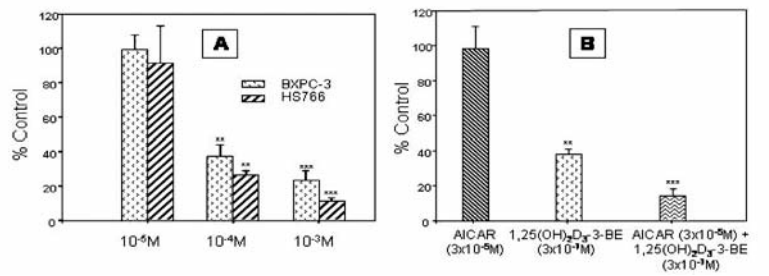

Figure 2. *A: Growth assays of BxPC-3 and HS766 cells, where cells were treated with various doses of AICAR or DMSO (control) on 1st, 3rd and 5th days. On the 7th day cells were trypsinized and counted in a hemocytometer. B: Growth assay of BxPC-3 cells treated with AICAR (3×10^{-5}M), or 1,25(OH)$_2$D$_3$-3-BE (3×10^{-7}M) or a mixture of AICAR (3×10^{-5}M), or 1,25(OH)$_2$D$_3$-3-BE (3×10^{-7}M) or DMSO (control) on 1st, 3rd and 5th days. On the 7th day, cells were trypsinized and counted in a hemocytometer. Results represent six replicates. Statistical analysis was carried out by Student's t-test (**p<0.01, ***p<0.001).*

AMPK is a serine/threonine protein kinase that is activated by AICAR (22). In order to determine whether its antiproliferative effect on pancreatic cancer cells is due to activation of AMPK and its down-stream target ACC, cell lysates from BxPC-3 cells, treated with AICAR were subjected to Western blot analysis. As shown in Figure 3A, both phopho-AMPK and phopho-ACC are up-regulated by AICAR in a dose-dependent manner, suggesting that growth inhibitory property of AICAR in BxPC-3 cells may be related to AMPK activation. On the other hand, when cells were treated with either AICAR (10^{-4}M) or 1,25(OH)$_2$D$_3$-3-BE (3×10^{-7}M) individually or in combination, phospho-AMPK level was strongly up-regulated by AICAR, while the same was reduced significantly compared to the control by 1,25(OH)$_2$D$_3$-3-BE (Figure 2B). The level of phospho-AMPK was partly restored by combining the two reagents. These results suggested that growth inhibition by AICAR and 1,25(OH)$_2$D$_3$-3-BE may either follow different pathways, or their observed enhanced activity in combination is not manifested *via* AMPK activation.

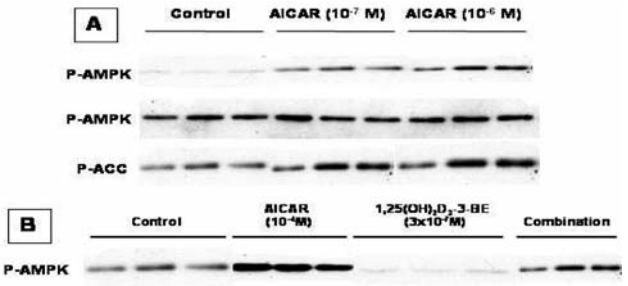

Figure 3. *A: Effect of AICAR (10^{-7}-10^{-6}M) on phosphorylation of AMPK and ACC in BxPC-3 cells. Cells were incubated with AICAR or DMSO control for 20 h, lysates were made and Western analysis was carried out with antibodies for phospho-AMPK and phospho-ACC. B: Effect of AICAR (10^{-4}M), 1,25(OH)$_2$D$_3$-3-BE (3×10^{-7}M), either individually or in combination on phosphoryaltion of AMPK. BxPC-3 cells were treated with various reagents or vehicle control for 20 h. Then lysates were made, and Western analysis was carried out with antibody for phospho-AMPK. The blots were stripped and re-probed for total AMPK as a loading control. These results are representative of two independent experiments.*

1,25(OH)$_2$D$_3$ is known to exert its cell-growth regulatory property *via* multiple direct and indirect cell-signaling pathways. In a direct effect, 1,25(OH)$_2$D$_3$ induces expression of cyclin-dependent kinase inhibitors (p15, p19, p21, p27), and inhibits G_0-G_1 transition (33, 34). On the other hand, there is evidence to suggest that the growth-inhibitory effect of 1,25(OH)$_2$D$_3$ and its analogs in certain cancer cells may involve up-regulation of p53 (14). In this study it was observed that p21 was up-regulated by 1,25(OH)$_2$D$_3$-3-BE, but not by AICAR, and combination of the two essentially reflects the level produced by 1,25(OH)$_2$D$_3$-3-BE alone (Figure 4, left panel). In contrast, the level of p53 was not different from the control when cells were treated with 1,25(OH)$_2$D$_3$-3-BE, while AICAR strongly up-regulated it. The combination, however, showed the strongest signal, suggesting that combined synergistic effect of 1,25(OH)$_2$D$_3$-3-BE and AICAR (observed in cellular studies) may reflect an increase in p53 regulation upon combining these reagents (Figure 4, left panel). It should be noted that in this assay, a higher concentration of 1,25(OH)$_2$D$_3$-3-BE (10^{-6}M) was used to obtain maximal response.

Akt (protein kinase B) is a serine/threonine kinase that is involved in signal transduction by the PI3K/Akt pathway (35, 36). Akt is involved in a variety of normal cellular functions. In addition, Akt has a profound effect in tumorigenesis, cell proliferation, growth and survival. Therefore, the regulation of Akt phosphorylation by AICAR and 1,25(OH)$_2$D$_3$-3-BE was evaluated, either alone or in combination in BxPC-3 cells. Results of the Western Blot analysis are shown in Figure 4, right panel. AICAR (6×10^{-5}M) strongly inhibited Akt phosphorylation, while 1,25(OH)$_2$D$_3$-3-BE (10^{-7}M) had a

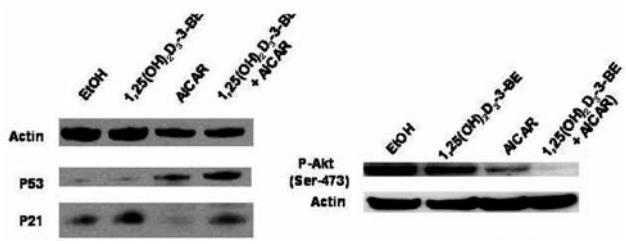

Figure 4. *Left panel: Effect of AICAR (3×10^{-5}M), 1,25(OH)$_2$D$_3$-3-BE (10^{-6}M), either individually or in combination on the expression of p21 and p53 in BxPC-3 cells. Right panel: Effect of AICAR (6×10^{-5}M), 1,25(OH)$_2$D$_3$-3-BE (10^{-7}M), either individually or in combination on the phosphorylation of Akt in BxPC-3 cells. Cells were treated with reagents or vehicle-control for 20 h, followed by making of lysates and Western Blot analysis in the usual fashion. β-Actin was used as a loading control. These results are representative of three independent experiments.*

considerably weaker effect. However, when the cells were dosed with a combination of the two reagents, Akt phosphorylation was almost completely eliminated. Strong inhibition of Akt phosphorylation by AICAR suggests growth inhibition by AICAR includes the PI3K/Akt pathway as well as AMPK activation, shown earlier. In contrast, nearly complete inhibition of Akt phosphorylation suggests that this pathway may be involved in explaining the increase in growth inhibition of PAC cells when these two reagents are combined.

In summary, results from this study demonstrate that 1,25(OH)$_2$D$_3$-3-BE, wether alone or in combination with AICAR, strongly inhibits the growth of several PAC cells, possibly *via* Akt/PI3K pathway. Furthermore, these results suggest a therapeutic potential for 1,25(OH)$_2$D$_3$-3-BE, alone or in combination with AICAR in PAC.

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