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Icilin induces G1 arrest through activating JNK and p38 kinase in a TRPM8-independent manner

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ABSTRACT

Aberrant regulation of cell cycle confers a limitless replicative potential, which is a hallmark of cancer. Currently, the compounds targeting the cell cycle are undergoing cancer clinical trials. In this study, we demonstrated that icilin, a cooling compound, induces G1 arrest in PC-3 prostate cancer cells without cell death. Icilin modulated the expression level of various cell cycle regulators at transcription or post-translational levels. In addition, icilin activated JNK and p38 kinase pathways, but not ERK. Both JNK and p38 kinases cooperatively mediated icilin-induced G1 arrest, which was rescued by pharmacologic inhibition of these kinases. The action of icilin on G1 arrest was unrelated to the activation of TRPM8 calcium channel. Our findings suggest that icilin is a valuable chemical probe for future investigation aiming at delineating the molecular mechanisms of cell cycle regulation in prostate cancer.

1. Introduction

Androgen-ablative therapy is an effective therapeutic regimen for treatment of advanced prostate cancer [1,2]. However, the prostate cancer eventually evolves into recurrent, incurable androgen-independent prostate cancer (AIPC) [3,4]. AIPC cells harbor many mutations in the cell cycle control genes [5,6], which are associated with the acquisition of an unlimited replicative potential in the androgen-depleted state. In addition, recent studies showed that androgen receptor interacts with various cell cycle regulators to drive deregulated cell cycle progression [5]. Therefore, targeting cell cycle may be a promising strategy for prostate cancer treatment [7,8]. In fact, many small molecule inhibitors against cell cycle regulators are currently undergoing cancer clinical trials [9,10].

Growing evidence suggests that certain cooling compounds, such as menthol, have therapeutic potentials in the treatment of several types of cancers, including prostate cancer [11]. These compounds stimulate Ca^{2+} mobilization across plasma membrane [12] or from intracellular stores [13] via multiple Ca^{2+} transport pathways, which is mediated by TRPM8 Ca^{2+} channel or unidentified off-target pathways [12,13]. However, it is unclear whether the antitumor effect of cooling agents is related to Ca^{2+} mobilization [14]. Moreover, little is known about the molecular mechanisms underlying the action of cooling agents. Furthermore, the chemotherapeutic activity of icilin, a synthetic supercooling compound, has not been explored at all.

MAPK family members are known to control cell cycle progression at various stages in a cell type- and context-specific manner [15]. A recent clinical study revealed that the phosphorylation of three members of MAPK family (ERK, JNK, and p38) significantly decreases in metastatic lesions when compared to primary prostate cancers [16]. This suggests that the decreased activity of MAPK members is associated with cell cycle deregulation in prostate cancer. However, the exact role of MAPK members in prostate cancer has been poorly understood. In this study, we present evidence that icilin has antiproliferative activity, which is unrelated to calcium transport pathways. We also demonstrate that icilin induces G1 arrest through activation of JNK and p38 kinase in PC-3, an AIPC model cell line. Our findings may provide novel insight into the understanding of prostate cancer biology.

Abbreviation: AIPC, androgen-independent prostate cancer.

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2. Materials and methods

2.1. MTT assay

PC-3 or HEK 293 cells were maintained under DMEM or RPMI medium containing 10% FBS (Hyclone) in 24-well culture plates. MTT assay was performed to assess cell growth according to the manufacturers' instructions (Sigma). The assay was quantitated by measuring the absorbance at 570 nm on microplate spectrophotometer (ASYS).

2.2. LDH release assay

Lactate dehydrogenase (LDH) release assay was employed to assess cell death according to the manufacturers' instructions (Promega). The assay was quantitated by determining the absorbance at 490 nm on microplate spectrophotometer.

2.3. Caspase-3 activity assay

Capase-3 activity was determined using a commercial kit according to the manufacturer's instructions (BioMol). The crude extract from PC-3 cells was used as a sample to analyze caspase-3 activity.

2.4. Intracellular Ca^{2+} measurement

Ratiometric fluorescence assay using Fura-2 dye was performed to determine intracellular calcium concentration ($[Ca^{2+}]_i$) as previously described [17]. $[Ca^{2+}]_i$ value was calculated from the equation, $[Ca^{2+}]_i = K_d \times \beta \times (R - R_{min})/(R_{max} - R)$ where K_d is the dissociation constant for Fura-2 (224 nM at 37 °C), β is F_{min}/F_{max}, and R is F_{340}/F_{380}. The autofluorescence signal of icilin was evaluated by the assays performed without labeling with Fura-2 dye.

2.5. Flow cytometry analysis

The cells were fixed with 70% ethanol and labeled with propidium iodide at 50 μg/ml (Sigma) containing RNase A at 100 μg/ml. The cell cycle phase was analyzed using flow cytometry (BD Biosciences). Annexin V staining was performed using FITC-conjugated annexin V (BD Biosciences) according to the manufacturer's instruction.

2.6. RT-PCR

Total RNA was extracted from LNCaP and PC-3 cells using TRIzol reagent (Invitrogen). Reverse transcription was performed using a commercial kit according to the manufacturer's instruction (Invitrogen). PCR was performed as described in Supplementary Fig. S1.

2.7. Western blot analysis

The total protein extracts were prepared by incubation with RIPA buffer containing protease and phosphatase inhibitor cocktails (Calbiochem). The protein samples were resolved in 8%, 10%, or 12% SDS–PAGE. Antibodies specific for PARP, pp38$^{T180/Y182}$,

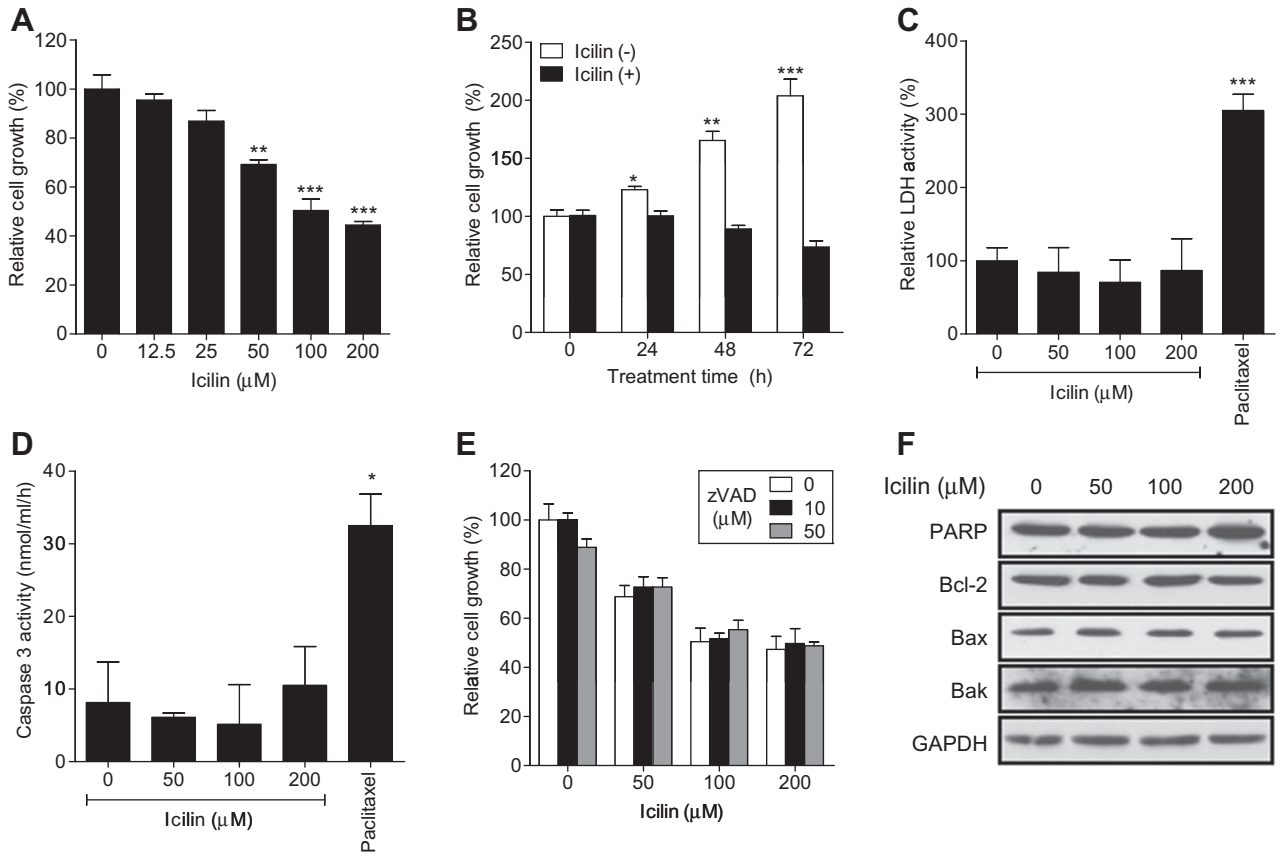

Fig. 1. Icilin induces growth arrest in PC-3 cells. The cells were treated with icilin at the indicated concentrations (A) or 200 M (B) for 72 h prior to MTT assays. Cell growth is expressed as a relative value to that of the untreated cells which is set to 100%. (C) The cells were treated with icilin at the indicated concentrations or paclitaxel as a positive control at 50 nM for 72 h prior to LDH release assays. LDH activity is expressed as a relative value to that of the untreated cells which is set to 100%. (D) The cells were incubated with icilin for 48 h prior to caspase-3 activity assays. The caspase-3 activity from untreated cells is expressed as 100%. (E) The cells were treated with icilin and/or zVAD-fmk at the denoted concentrations for 72 h prior to MTT assays. (F) Western blot analysis was performed using the crude extract from PC-3 cells following treatment with icilin at the indicated concentrations for 72 h. The figures show mean ± SEM ($n = 4–6$). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$.

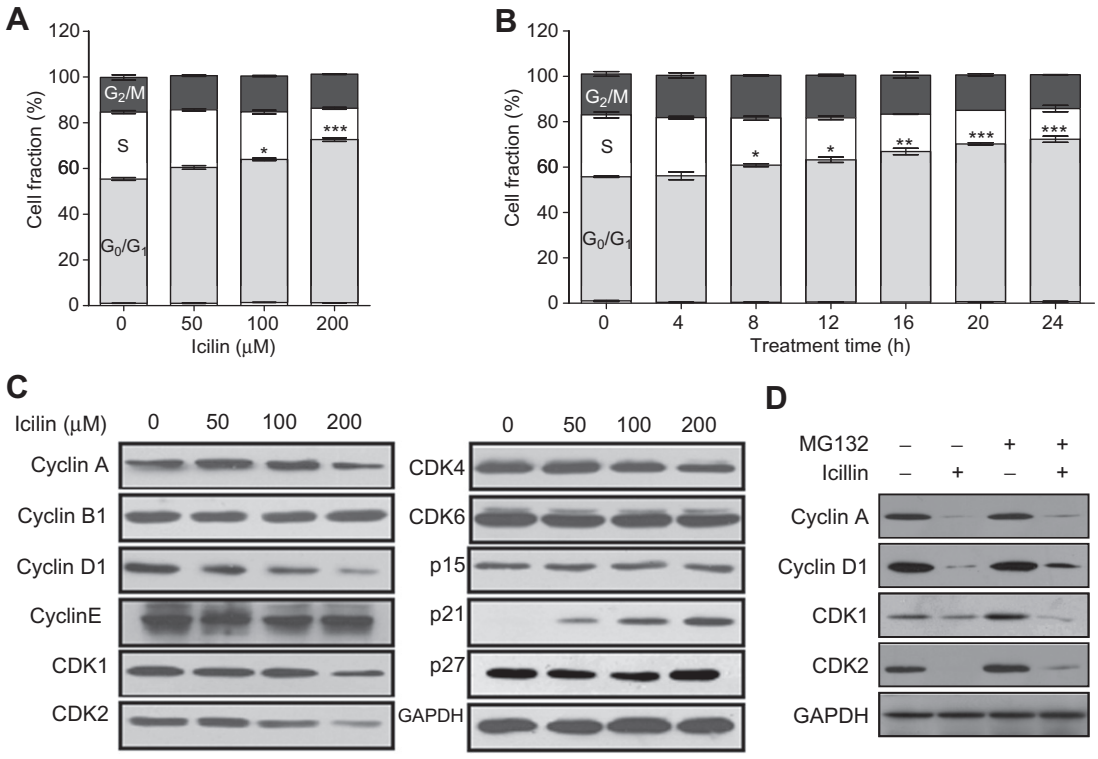

Fig. 2. Icilin induces G1 arrest in PC-3 cells. The cells were incubated with icilin at the denoted concentrations (A) or 200 μM (B) for 24 h prior to cell cycle analysis. Cell fraction is expressed as the percentage of cells in each phase of the cell cycle. (C) Western blot analysis was performed using the crude extract from PC-3 cells following treatment with icilin at the indicated concentrations for 24 h. (D) The cells were treated with icilin at 200 μM and/or MG132 at 5 μM. The figures show mean ± SEM ($n = 4–6$). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$.

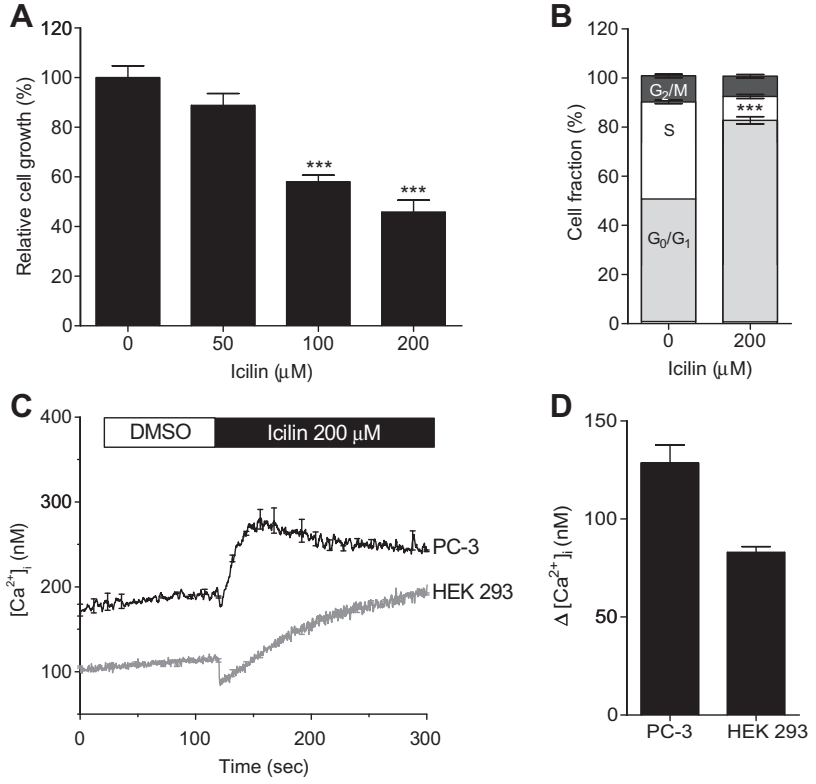

Fig. 3. Icilin-induced G1 arrest is independent of TRPM8 activation. (A) HEK 293 cells were treated with icilin at the indicated concentrations prior to MTT assays. (B) HEK 293 cells were incubated with icilin at 200 μM for 24 h prior to cell cycle analysis. The $[Ca^{2+}]_i$ change pattern (C) or the difference in peak $[Ca^{2+}]_i$ increase (D) evoked by icilin at 200 μM were quantitated in PC-3 or HEK 293 cells. The figures show mean ± SEM ($n = 4–6$). $^{***}p < 0.005$.

p38, pJNK$^{T183/Y185}$, JNK, pERK$^{T202/Y204}$, ERK, Cyclin A, Cyclin B1, Cyclin D1, Cyclin E, CDK1, CDK4, CDK6, p15, p27, pMKK3/6$^{S189/207}$, MKK3/6, pATF-2$^{T69/71}$, ATF-2, pSEK1/MKK4$^{S257/T261}$, pc-Jun^{S63}, c-Jun, pMnk1$^{T197/202}$, pMAPKAPK-2^{T334}, MAPKAPK-2, pMSK1^{S376}, and MSK1 were supplied by Cell Signaling. Antibodies to p21, Bcl-2, Bax, Bak, Mnk1, and GAPDH were purchased from Santa Cruz.

2.8. Statistic analysis

All data presented are expressed as mean ± SEM. Comparison of mean values among experimental groups was performed with ANOVA followed by a post hoc test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Icilin arrests the growth of PC-3 cells

To explore whether icilin has antiproliferative activity, we performed MTT assay with PC-3 cells. Cell population was gradually reduced in relation to icilin concentrations (Fig. 1A). However, massive cell death was not observed under the experimental conditions (data not shown), which were further corroborated by quantitating cell growth over time (Fig. 1B). Thus, the antiproliferative activity of icilin is ascribed to growth arrest rather than cell death. This result was ascertained by a series of experiments. First, icilin elevated neither LDH nor caspase-3 activity, compared to paclitaxel, a chemotherapy agent (Fig. 1C and D). Second, icilin did not increase the cell population stained with annexin V-conjugated FITC (Fig. S1). Third, pan-caspase inhibitor zVAD-fmk did not rescue icilin-induced growth inhibition (Fig. 1E). The functionality of zVAD-fmk was confirmed by caspase-3 activity assay (Fig. S2). Finally, icilin did not cause the changes associated with apoptosis-related molecules, such as PARP, Bcl-2, Bax, and Bak. Therefore, these data demonstrate that icilin has the ability to induce growth arrest without triggering cell death.

3.2. Icilin induces G1 arrest in PC-3 cells

To understand the mechanisms of icilin action, we analyzed cell cycle profile of PC-3 cells. Flow cytometric analysis showed that the percentage of G1 phase cells markedly increases in the cells treated with icilin (Fig. 2A). A significant increase in G1 phase cell fraction was observed following treatment with icilin at 200 μM for 8 h and further augmented thereafter (Fig. 2B). We then examined the molecular changes associated with cell cycle regulation. Western blot analysis showed that the expression levels of cyclin A, cyclin D1, CDK1, and CDK2 are down-regulated by icilin, whereas p21 is up-regulated (Fig. 2C). By contrast, the expression levels of cyclin B1, cyclin E, CDK4, CDK6, p15 and p27 were not affected by icilin. These results indicate that icilin induces G1 arrest via selectively modulating the expression of a subset of cell cycle regulators.

We then questioned the underlying mechanisms by which icilin regulates the expression of a subset of cell cycle regulators. RT-PCR

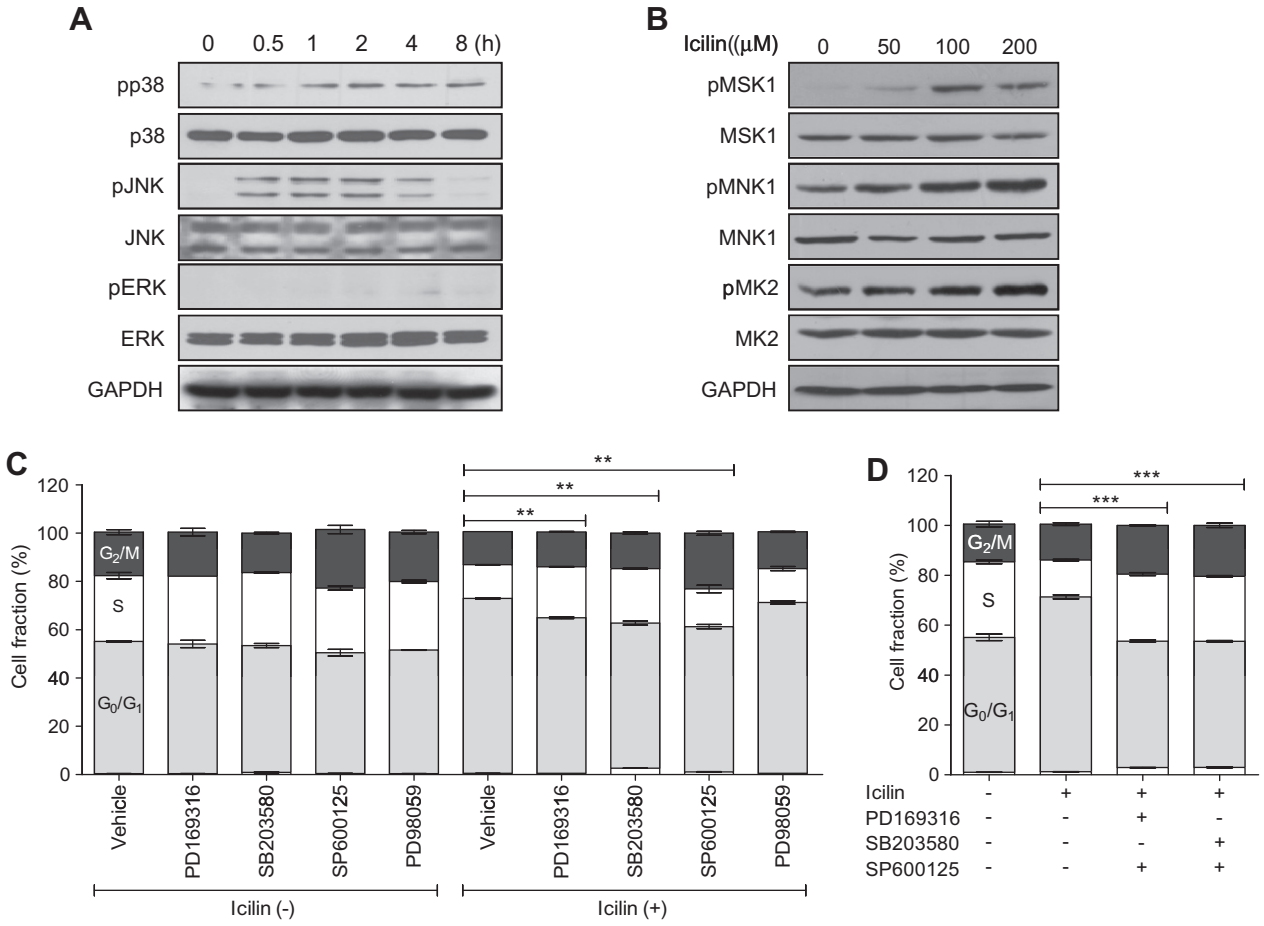

Fig. 4. Icilin-induced G1 arrest is independent of TRPM8 activation. (A) HEK 293 cells were treated with icilin at the indicated concentrations prior to MTT assays. (B) HEK 293 cells were incubated with icilin at 200 μM for 24 h prior to cell cycle analysis. The $[Ca^{2+}]_i$ change pattern (C) or the difference in peak $[Ca^{2+}]_i$ increase (D) evoked by icilin at 200 μM were quantitated in PC-3 or HEK 293 cells. The figures show mean ± SEM ($n = 4–6$). $^{***}p < 0.005$.

analysis showed that icilin down-regulates CDK1 and CDK2 but up-regulates p21 (Fig. S3). The RNA levels of cyclin A and D1 were little changed by icilin. Western blot analysis showed that the protein level of only cyclin D1 was rescued by MG132, a proteosomal inhibitor (Fig. 2D). These results suggest that icilin controls CDK1, CDK2, and p21 at transcriptional level, cyclin D1 at post-translational level, and cyclin A at translational level, providing the insight into the mechanisms of action of icilin.

3.3. Icilin-induced growth arrest is unrelated to TRPM8

Because icilin is a well-known agonist of TRPM8 [12], we examined whether icilin is able to induce growth arrest in HEK 293, a TRPM8-negative cell line [13,14]. MTT assays showed that icilin attenuates the growth of HEK 293 cells in a dose-dependent manner (Fig. 3A). The percentage of G1 phase cells markedly increased in the cells treated with icilin (Fig. 3B). We then measured the calcium changes in response to icilin. Although the $[Ca^{2+}]_i$ of HEK 293 cells slightly increased, the change was substantially smaller than that of PC-3 cells (Fig. 3C and D). Nonetheless, icilin noticeably arrests the growth of HEK293 cells, showing that the extent of Ca^{2+} changes does not correlate with that of growth inhibition. Because TRPM8 is not expressed in the cells, the increase of $[Ca^{2+}]_i$ in HEK293 cells was ascribed to the off-target effect of icilin. These observations indicate that icilin induces growth arrest irrespective of TRPM8 activation.

3.4. JNK and p38 kinase mediate icilin-induced G1 arrest in PC-3 cells

Because MAPK family members play a crucial role in cell cycle regulation [16,17], we examined the change of MAPK activity in PC-3 cells following treatment with icilin. Western blot analysis showed that the activity of JNK and p38 kinase is elevated by icilin, whereas that of ERK is not changed (Fig. 4A). In addition, we observed increased phosphorylation of MKK3/6 (p38 kinase upstream), ATF-2 (p38 kinase downstream), MKK4 (JNK upstream), and c-Jun (JNK downstream) (Fig. S4), confirming that icilin activates both JNK and p38 signaling pathways. Furthermore, we found that icilin caused the increased phosphorylation of MK2, MSK1, and MNK1, which are the p38 downstream molecules involved in cell cycle arrest or apoptosis [18].

We then investigated whether JNK and p38 kinase are causally related to icilin-induced G1 arrest using 10 μM of PD169316 (p38 inhibitor), SB203580 (p38 inhibitor), SP600125 (JNK inhibitor), or PD98059 (ERK inhibitor). Each agent *per se* did not induce significant changes in cell cycle profile (Fig. 4C). On the other hand, PD169316, SB203580, and SP600125, but not PD98059, partly rescued icilin-induced G1 arrest (Fig. 4C). When the cells were co-treated with JNK and p38 kinase inhibitors, cell cycle profile was completely restored to normal profile (Fig. 4D). These results demonstrate that co-activation of JNK and p38 kinase play a crucial role in icilin-induced G1 arrest pathways.

4. Discussion

Deregulation of cell cycle is a hallmark of cancers, which is linked to uncontrolled proliferation of tumor cells [19]. AIPC cells accumulate mutations in androgen receptor to allow the cells to survive in the androgen-depleted conditions [20]. Recently, androgen receptor is found to interact with various cell cycle regulators [5], indicating that androgen receptor plays a crucial role in cell cycle control. These observations suggest that cell cycle-targeted therapy can be a promising strategy for treatment of prostate cancer. In this study, we demonstrate that icilin induces G1 arrest without cell death via activating JNK and p38 kinase in PC-3 cells. Therefore, our findings suggest that icilin is a useful compound for future development as an anticancer agent.

Various CDK inhibitors that enter cancer clinical trials target the ATP-binding site of CDK molecules [21]. Unlike other CDK inhibitors, icilin inhibits CDKs by reducing the expression level of cyclins or CDKs. In addition, several CDK inhibitors induce apoptosis [21], whereas icilin does not trigger apoptosis. There are three potential reasons for the phenotypic difference: (1) other CDK inhibitors elicit off-target responses to induce apoptosis, (2) CDK inhibitory activity of icilin is insufficient to trigger apoptosis, or (3) CDK isotypes that is affected by icilin are unrelated to apoptosis. Further study on the anti-proliferative mechanisms of icilin will provide novel insight into the development of anticancer strategies.

JNK and p38 kinase can exert both tumor suppressive and oncogenic functions in a cell type-specific or cellular context-specific manner [16]. The molecular mechanisms by which JNK and p38 kinase determine cellular fate are poorly understood, particularly in prostate cancer. In addition, the crosstalk between these kinases and resulting phenotypic consequences are largely unknown. Our data show that JNK and p38 kinase cooperatively regulate cell cycle progression. Particularly, icilin activated MSK1, MNK1, and MK2 that mediate the tumor-suppressive function of p38 [22–25]. Thus, we propose that icilin is a promising chemical probe to assist in understanding the role of JNK and p38 kinase in cell cycle regulation. One of future challenges is to elucidate the molecular relationship between the activity of JNK/p38 kinase and the expression of cell cycle regulators. In addition, it is further needed to investigate whether the G1 arrest mediated by JNK and p38 kinase may be the adaptive responses of the tumor cells exposed to cytotoxic stress.

We show that icilin is able to induce G1 arrest in TRPM8-negative HEK293 cells. These results indicate that the off-target effect of icilin mediates G1 arrest, which also suggest that icilin-induced JNK and p38 activation is independent of TRPM8 activation. In addition, EC_{50} values for icilin in TRPM8 activation were reported to be approximately 0.2 μM [26], which further indicates that TRPM8 is not involved in the action of icilin on G1 arrest. Therefore, the identity of the proximal targets of icilin is needed to be determined.

In summary, we demonstrate that icilin induces G1 arrest by activating JNK and p38 kinase independent of TRMP8 activation. Our findings indicate that icilin would be a valuable chemical probe for future investigation aiming at illuminating the molecular regulatory mechanisms underlying cell cycle regulation in prostate cancer.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.01.094.

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