CR389, a Benzoimidazolyl Pyridinone Analog, Induces Cell Cycle Arrest and Apoptosis via p53 Activation in Human Ovarian Cancer PA-1 Cells

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Serine/threonine kinases play crucial roles in regulating a wide range of cellular responses, such as cell proliferation and differentiation of eukaryotic cells [7, 13]. The cyclin-dependent kinases (CDKs), which belong to this family, are pivotal regulators of cell cycle progression in cooperation with specific endogenous cyclins and CDK inhibitors, including p21CIP1 and p16INK4a [11, 21]. Achieving cell cycle control is an ultimate goal in the treatment of diseases characterized by uncontrolled cell proliferation [4, 19]. Consequently, the CDKs are important molecular targets for drug discovery, and the inhibition of CDKs may be particularly useful in the treatment of cancers [3, 5, 6, 8, 12, 15–18].

Apoptosis, which can be induced through either the stimulation of the death receptor or the release of cytochrome c from mitochondria, has been characterized as a fundamental cellular event to maintain tissue homeostasis in eukaryotic organisms [10]. Therefore, an imbalance between growth-promoting and growth-inhibiting mechanisms through deregulated cell cycle progression and impaired apoptotic induction is involved in neoplastic cancer formation [10, 14].

The p53 tumor-suppressor protein is a tightly regulated transcription factor that can induce either cell cycle arrest or apoptosis according to its expression and activation level. The p53 protein normally responds to different forms of cellular stress by binding the consensus sites of target genes, such as p21CIP1 and pro-apoptotic Bax protein, that inhibit cell cycle progression and trigger apoptotic cell death, respectively [1, 20]. Playing such a pivotal part in controlling cell growth and survival, p53 has earned the title of “Master executioner.”

To generate new scaffold candidates as potent CDK inhibitors, structure-based drug screening was performed using the X-ray crystallographic structure of CDK2. As a result, 5-(1H-benzoimidazol-2-yl)-1H-pyridin-2-one, designated as CR389, was identified as a hit compound.

In the present study, we demonstrate in human ovarian cancer...
cancer PA-1 cells that CR389 (Fig. 1A) induces cell cycle arrest at the G2/M phase via up-regulation of p21CIP1 and apoptosis through the cytochrome c-mediated intrinsic pathway, both effects of which result from the activation of the p53 tumor-suppressor protein.

To examine whether CR389 can inhibit CDK activity, we used in vitro kinase assays employing the peptide substrate histone H1 for CDK1 and CDK2. As shown in Fig. 1B, CR389 inhibited CDK1 and CDK2 in a dose-dependent manner, with IC50 values of 47.5 and 58.6 µM, respectively.

**Fig. 2.** (A) Quantitative flow cytometric histogram of PA-1 cells treated with 40 µM CR389 and (B) flow cytometric analysis of CR389-treated PA-1 cells with synchronization of the cells in the G1 phase using thymidine.

PA-1 cells were treated with 10 mM thymidine for 8 h. Then, the cells were washed three times and treated with 40 µM CR389 for the indicated time. The cells were stained with propidium iodide and the nuclei were analyzed for their DNA content by flow cytometry using CellQuest software. A total of 10,000 nuclei were analyzed from each sample.
Thus, it could be concluded that CR389 induces cell cycle arrest at the G2/M phase in PA-1 cells through direct inhibition of CDK1 and CDK2.

To examine the effect of CR389 on cell cycle progression in PA-1 cells, we measured the DNA content of PA-1 cells treated with 40 µM CR389, the IC$_{50}$ value obtained from the MTT assay, using flow cytometric analysis. As shown in Fig. 2A, the PA-1 cell population gradually increased from 26% at 0 h to 35% at 12 h in the G2/M phase after exposure to 40 µM CR389.

To rule out the possibility that CR389 could be associated with specific apoptotic induction of cells arrested only at the G1 phase, we used thymidine, a DNA polymerase inhibitor, which arrests cells in the G1 phase without any cytotoxicity at a concentration of 10 mM. If thymidine is used to treat PA-1 cells, the G1 phase population is dramatically enhanced (typically, more than 65%). As shown in Fig. 2B, the percentage of the cell population in the G2/M phase was elevated from 10% at 0 h to 67% at 12 h, and the number of cells in the G1 phase decreased dramatically from 71% at 0 h to 13% at 12 h. Therefore, it could be concluded that PA-1 cells treated with 40 µM CR389 showed an appreciable G2/M phase arrest. Furthermore, the sub-G1 phase population, that is, the apoptotic population (AP), increased significantly up to 38% at 24 h after treatment with 40 µM CR389.

To investigate the apoptotic induction of CR389 in PA-1 cells, a TUNEL assay was used to examine DNA fragmentation in PA-1 nuclei. As shown in Fig. 3A, apoptotic induction was identified in CR389-treated PA-1 cells in a time-dependent manner for 48 h. Furthermore, the formation of fragmented apoptotic bodies, which are typically observed in apoptosis, was obviously induced after 24 h of treatment with 40 µM CR389 (Fig. 3B).

In addition, to determine whether apoptosis-related proteins are involved in the mediation of CR389-induced cell death in PA-1 cells, we examined the activation of procaspases and cleavage of PARP by western blot analysis. As shown in Fig. 4, total cell lysates from PA-1 cells treated with 40 µM CR389 for the indicated time were electrophoretically separated on an 8–14% polyacrylamide gel and immunoblotted with antibodies against target proteins. β-actin was used as a loading standard.
in Fig. 4A, CR389 induced the proteolytic cleavage of inactive procaspase-9 and -3 into active caspase-9 and -3, respectively. Furthermore, one of the substrates for effector caspases, such as caspase-3, is PARP, an enzyme that appears to be involved in DNA repair and genome surveillance in response to environmental stress. Thus, cleavage of PARP was used as an indicator of apoptotic induction, which became obvious after 24 h of CR389 treatment. Furthermore, we found that CR389-induced apoptosis was associated with the increased expression of pro-apoptotic protein Bax, which is one of the downstream target genes of p53 (Fig. 4B).

Post-translational modification of the p53 tumor-suppressor protein by phosphorylation at Ser15 has been proposed to be a crucial mechanism by which p53 is stabilized and activated. As shown in Fig. 4B, phosphorylation of p53 at Ser15, designated as pp53Ser15, was apparently increased in a time-dependent manner in 40 µM CR389-treated PA-1 cells. Furthermore, we also found that cell cycle arrest in CR389-treated cells was associated with increased expression of p21^{CIP1}, which is a downstream target gene of p53 and a key regulator for the entry of cells at the G2/M transition checkpoint [2, 9, 22]. Thus, it could be concluded that cell cycle arrest at the G2/M phase via up-regulation of p21^{CIP1} and Bax-mediated apoptotic induction in CR389-treated PA-1 cells resulted from the stabilization and activation of p53.

In conclusion, to examine the Bax-mediated death pathway involved in CR389-induced apoptosis, we analyzed cytochrome c release from mitochondria to the cytosol using confocal laser scanning microscopy. As shown in Fig. 5, cytochrome c (green) release was observed significantly in 40 µM CR389-treated PA-1 cells. However, cytochrome c release from mitochondria in non-treated control cells was not observed. Therefore, we concluded that cytochrome c release is obviously induced by treatment with CR389.

In addition, to examine the Bax-mediated death pathway involved in CR389-induced apoptosis, we analyzed cytochrome c release from mitochondria to the cytosol using confocal laser scanning microscopy. As shown in Fig. 5, cytochrome c (green) release was observed significantly in 40 µM CR389-treated PA-1 cells. However, cytochrome c release from mitochondria in non-treated control cells was not observed. Therefore, we concluded that cytochrome c release is obviously induced by treatment with CR389.

In conclusion, we demonstrated that treatment with CR389 in PA-1 cells is associated with an accumulation of cell populations in the G2/M phase via up-regulation of p21^{CIP1} and with apoptotic induction through the cytochrome c-mediated intrinsic pathway, both effects of which are induced by a key molecular event, the activation of p53. Accordingly, CR389 seems to have multiple mechanisms of antiproliferative activity through p53-mediated pathways against human ovarian cancer cells. More detailed studies are needed to establish both the cancer preventive and therapeutic efficacy of CR389 in this and other types of cancer cells.

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