Characterization of the Ubiquitination of ICER, a Potential Anti-Cancer Protein, in Human Melanoma Cells

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MONTCLAIR STATE UNIVERSITY

CHARACTERIZATION OF THE UBQUITINATION OF ICER, A POTENTIAL ANTI-CANCER PROTEIN, IN HUMAN MELANOMA CELLS/

by

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Inducible cAMP Early Repressor (ICER) is a transcriptional repressor that regulates the expression of cAMP inducible genes. ICER has recently garnered attention because of cAMP’s implication in oncogenesis. ICER is shown to be downregulated in melanomas by the means of the ubiquitin-proteasome pathway. It is marked for degradation by ubiquitination on its lysine residues. By rescuing ICER levels in cancer cells, it is hypothesized that it may be possible to reverse the adverse progression of melanoma tumor growth. One possible way of rescuing ICER is by making it unavailable for ubiquitination by altering its ubiquitination sites. Special mutant ICERs that have their lysines mutated may offer the answer. Consequently, due to its nature as a transcription factor, ICER must be localized in the nucleus in order to function properly. Through immunocytochemistry, Western blotting, luciferase assay, and TUNEL apoptosis analyses of SK-MEL-24 cancer cell lysine knockout mutants, we were able to show strong nuclear subcellular localization of our mutant ICERs and efficient binding to DNA, a requirement for a transcription factor. This could potentially lead to further study of ICER as a cancer treatment.
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Introduction

Inducible cAMP Early Repressor (ICER) is a very small transcriptional repressor that regulates the expression of cAMP inducible genes. "ICER" is a general term for various isoforms ranging in size from 108 to 120 amino acids and resulting from the CREM (cAMP Responsive Element Modulator) gene. This protein functions as a homodimer to strongly bind DNA and prevent it from being transcribed, effectively silencing the genes it regulates. ICER has two specific domains, an N-terminal basic leucine zipper domain and a C-terminal DNA binding domain. The latter is known to bind to the cAMP response element, or CRE, which is a canonical DNA sequence, TGACGTCA, located in cAMP-regulated gene promoters.¹

![Figure 1. 3D Model of CREB Isoform. A three-dimensional representation of a CREM gene isoform binding to DNA as a homodimer.²](image-url)
ICER is strongly conserved between species that have it which underlies the importance of its function and has become of interest due to cAMP's implication in oncogenesis. Cyclic AMP helps to regulate growth and differentiation of melanocytes, or melanin producing epidermal cells, which are responsible for the development of human melanoma. One type of ICER, isoform ICER-I □, has already been shown to be absent from the nucleus in various cancer cell types, including melanoma. On the other hand, ICER levels are strongly elevated in cases of degenerating melanoma cells. This leads to the possibility that ICER may potentially play a role in the destruction of melanoma in humans. In cases of melanoma, ICER is either removed from the nucleus or degraded via ubiquitination. Since ubiquitination is dependent on ubiquitin being covalently bound to lysine residues on target proteins, we hypothesize that eliminating ubiquitination sites may hold the key to rescuing endogenous ICER levels in these cells.

As a result, recent work has been done to try and establish ICER as a tumor suppressor protein. This is due to the phenomena in cancer cells where ICER levels are much lower than that of healthy cells. Rescuing ICER levels in these depleted cancer cells inhibits growth and negatively impacts the traits that enable cancer cells to grow uncontrollably such as rampant DNA synthesis and the upregulation of cell cycle related genes. The pathway through which ICER is degraded in cancer cells has also been elucidated and is facilitated by the use of the post-translational modifier protein ubiquitin. In cancers, ICER is either monoubiquitinated, which causes it to be sequestered outside the nucleus rendering it useless, or it is polyubiquitinated, which causes it to be degraded through the ubiquitination-proteasome pathway. In order to be affected by these two pathways, ICER needs to be able to be phosphorylated and...
ubiquitinated. Phosphorylation marks ICER for ubiquitination and takes place on serine residues within its primary sequence. It has been shown that in the absence of the serine residues, ICER levels are higher than when compared to the wildtype. However, ubiquitination itself takes place on its lysine residues. Potentially, this means that mutating the various lysine residues of ICER into a chemically similar amino acid, such as arginine can potentially reduce or eliminate ICER’s ability to be ubiquitinated and degraded.\(^8\)

![Lysine and Arginine](image-url)

**Figure 2. Amino Acids Lysine and Arginine.** Both lysine and arginine are polar, positively charged amino acids and, under usual circumstances, can be substituted for each other without functional protein consequences being observed.

Melanoma is the sixth most common cancer in the United States.\(^9\) The prognosis for which is very good if the tumor is caught early. However, if the melanoma becomes metastatic and spreads to other parts of the body before treatment has started, the rate of survival is dramatically reduced.\(^10\) Studies have shown that the oncogenes \textit{BRAF} and \textit{RAS} are responsible for at least 66% of all malignant melanoma cases.\(^11\) A mutation in the \textit{BRAF} gene, specifically, V600E, contributes to significant upregulation in the mitogen-activated protein kinase (MAPK) pathway. This is significant because ICER is
involved in the MAPK pathway, which, in turn, is involved in cell division and the
development of melanoma. Specifically, when this pathway is down regulated, ICER is
stabilized by cAMP.\textsuperscript{12} Alternatively, a consequence of this pathway activation is the
phosphorylation and ubiquitination of ICER.\textsuperscript{13} Additionally, forced expression of ICER
results in an inability for cancer cells to grow in an anchorage-independent manner,
resulting in inhibited tumor growth.\textsuperscript{14} Expression of ICER has been linked to
improvement in other types of cancers, such as leukemia.\textsuperscript{15} Exclusion of ICER in these
cases may contribute to the symptoms associated with metastatic melanoma. Logically,
this leads to the conclusion that rescuing ICER levels in cancerous cells may lead to
apoptotic death in the affected cells.

The ultimate goal of this study is to determine whether or not various ICER-lysine
mutants would still be degraded via the same pathway or if they would persist and
maintain their original function and location in the nucleus of melanoma cells. If the
mutants do persist, the next step is to investigate the apoptotic effect of ICER on these
cells.
Materials and Methods

Cell Transfections for Immunocytochemistry, Western Blot, Luciferase, and TUNEL

SK-MEL-24 cells were purchased (ATCC) and grown using the Nunc Lab-Tek Chamber slide system (Thermo Scientific) until they were 80% confluent. The cells are anchorage dependent and adhere to the bottom of the slides. The chambers allowed for separation of the cells into distinct populations that would be differentiated based on their respective ICER constructs. FuGENE HD Transfection Reagent (Promega) was the system utilized to transfect the populations. Individual ICER mutant constructs were diluted to ~1 μg/μL and mixed with the appropriate amount of FuGENE reagent as per the protocol’s recommendation in order to form the transfection complex. Once formed, the complex was administered to the cells. The total volume of each slide chamber was brought up to 25 μL using cell media and left to incubate over 48 hours. The protocol for each transfection was the same with the only variable being the proportions of the reagents.

Immunocytochemistry

The cells were grown and transfected as described above. The culture medium was removed and the cells were washed two times with phosphate buffered saline (PBS) at room temperature. The cells were fixed by adding ~1 mL 4% formaldehyde in PBS into the chambers and left for 20 minutes. The formaldehyde was then aspirated and the cells were washed with PBS three times for 5 minutes each then stored for several days. Two days later, the antigen retrieval buffer was preheated to 95 degrees Celsius and 1 mL
of the buffer was added to each chamber and the slides were incubated at 95 degrees Celsius for 10 minutes. The buffer was aspirated and the cells washed three times with PBS. The cells were then incubated in 0.1% Triton X-100 in PBS for 15 minutes at room temperature then washed three times with PBS. All remaining PBS was removed and the protein block was added and the cells were left to incubate for 1 hour at room temperature. Phosphate buffer saline was used to wash the cells again and the primary antibody (polyclonal Rabbit anti-HA at 1 μg/mL in blocking buffer) was applied and cells left to incubate overnight at 4 degrees Celsius. The next day, the cells were washed four times with PBS and the secondary antibody (biotinylated Goat anti-Rabbit Polyvalent - Abcam) was applied and the cells left to incubate for 10 minutes at room temperature. The cells were again washed four times with PBS then treated with streptavidin peroxidase and left to incubate for another 10 minutes at room temperature. The cells were then washed four more times with PBS then treated with 30 μL of DAB Chromogen and 1.5 mL of DAB Substrate and swirled. After a final incubation for ~10 minutes and wash with PBS, a drop of mounting medium was applied and the results observed.

**Nuclear and Cytosolic Fractionation**

SK-MEL-24 cells were grown in chambers and transfected as described above. The culture medium was then aspirated and the cells were washed with 2 mL of PBS at room temperature. Next, the cells were harvested by adding 750 μL of trypsin-EDTA and incubated at 37 degrees Celsius for three minutes. Then 750 μL/mL of complete cell culture media was added and gently pipetted up and down to release the cells from the chamber. The cells were transferred to 1.5 mL microcentrifuge tubes and centrifuged at
500 x g for 5 minutes. Afterwards, the pellets were suspended with 1 mL of ice-cold PBS and centrifuged again at 500 x g for 2.5 minutes. The supernatant was discarded and 100 μL of ice-cold Cytosolic Extraction Reagent I (CER I) was added. The tubes were vortexed on high for 15 seconds to resuspend the pellet and then were incubated on ice for 10 minutes. Then, 5.5 μL of ice-cold CER II was added to the tubes and the tubes were vortexed again on high for 5 seconds and put on ice for 1 minute. Next, the tubes were centrifuged for 5 minutes at ~16,000 x g. The supernatant (cytosolic extract) was transferred to clean, cold tubes and stored at -80 degrees Celsius. The pellet was resuspended in 50 μL of ice-cold Nuclear Extraction Reagent (NER). The tubes were then vortexed on high for 15 seconds every 10 minutes for 40 minutes. The tubes were centrifuged again at ~16,000 x g for 10 minutes. The resulting supernatant (nuclear extract) was transferred to clean, cold tubes and stored at -80 degrees Celsius.

**Western Blot and IR Detection**

The protein extracts from the previous sections were analyzed via Western blot. Ten μL of the extracts were pipetted into each lane of two sets of polyacrylamide-SDS gels and ran at a constant 200 V for 45 minutes. The first set of gels was probed for ICER while the next set was probed for beta actin as a loading control. The protein on the resulting gel was transferred onto a nitrocellulose membrane at a constant 100 V for one hour. Nonspecific antibody protein binding was blocked by incubating the membranes in blocking buffer for 15 minutes. For the ICER membranes, the primary rabbit anti-HA antibody was added to a 1 μg/mL solution of blocking buffer and incubated at room temperature for 6 hours on a swirling platform. The membranes were then washed four
times for 5 minutes each with PBS containing 0.1% Tween 20 at room temperature. The secondary goat anti-rabbit antibody was applied in a 1:10,000 dilution in blocking buffer and the membranes left to incubate at room temperature for 45 minutes. Again, the membranes were washed four times for 5 minutes each with PBS containing 0.1% Tween 20. The protocol was then repeated for the beta-actin membranes, using anti-beta actin as the sole antibody. The membranes were then analyzed using near-infrared fluorescence detection on the Odyssey Imaging System.

*Dual Luciferase Reporter Assay*

SK-MEL-24 cells were grown in five, twenty-four well dishes in 0.5 mL of media per dish. Next, 2 mL of 0.25% trypsin was added to each well for incubated for 5 minutes at 37 degrees Celsius and 8 mL of media was added in addition. The cells were then transfected as described above. The culture medium was then aspirated and the cells were washed with PBS and each well treated with 100 μL of passive lysis buffer. The cells were then rocked gently for 15 minutes at room temperature and lysate was transferred to new tubes for testing. To activate firefly luciferase, 100 μL of the luciferase assay reagent II was added to each tube and the luminometer took a reading five seconds later for about ten seconds each. To activate Renilla luciferase, 100 μL of Stop & Glo reagent (Promega) was added and measured by luminometer in the same fashion. This was repeated for each tube.
Terminal Deoxynucleotidyl Transferase (TdT) dUTP Nick-End Labeling Assay

SK-MEL-24 cells were grown in chambers and transfected as described above. The culture medium was then aspirated and the cells were fixed in 4% formaldehyde in PBS for 25 minutes at 4 degrees Celsius. Next, the slides were immersed in PBS twice, 5 minutes each time. The slides were then immersed in 0.2% Triton X-100 in PBS for 5 minutes and washed by immersion twice in PBS, 5 minutes each time. Twenty-five μL of equilibration buffer was added to the cells at left to incubate at room temperature for 10 minutes. The cells were then labeled using 25 μL of TdT reaction mixture to each population and covered with a cover slip, then incubated in a humidified chamber at 37 degrees Celsius for 1 hour. Afterwards, the slides were immersed in SSC for fifteen minutes to stop the reaction. PBS was then used to wash the slides by immersion three times for 5 minutes each. The cells were counterstained with DAPI and analyzed by fluorescent microscopy.
Results

Immunocytochemistry

We utilized immunocytochemistry in order to determine the subcellular localization of our mutant ICERs. Nuclear localization of ICER is required for the protein to fulfill its function. The results for the GFP control for each one of the slides shows very little, if any, ICER signal. Specifically, three out of the five controls showed no significant signal while the other two show a relatively light and even distribution of ICER throughout both the nucleus and the cytoplasm (Figures 3-7). When analyzing the ICER mutants, it is important to note the noticeable difference in the intensity of the ICER signal when comparing to the controls. Under normal circumstances, ICER would be either present in the nucleus or presumably ubiquitinated and sequestered to the cytoplasm. However, almost every ICER mutant shows considerable presence in the nucleus and cytoplasm with a strong bias towards the nucleus.

The mutants located in the N-terminal domain, K0-52 through K0-70 (Figure 4), of the protein may have less of a dramatic effect on ICER binding and ubiquitination. These cells show an ICER signal more prominently than the control but not as much as the mutants located in the DNA binding domain. K0-76 through K0-97 (Figures 5-6), which happen to be located in the DNA binding domain of ICER, have the highest nuclear to cytosolic ratio in terms of signal out of the mutants tested, even more so than the complete K0 mutants. Interesting though, the HA tagged wildtype cells show a convincingly even distribution of ICER throughout. The wildtype cells would be expected to more closely resemble the controls than the mutants.
Figure 3. Micrographs of the Control, Nwt, CKO, and Cwt. The GFP control is showing a neutral, blank signal throughout all the cells in the field of view. CKO, which has an HA tag on the C terminus, shows a strong nuclear signal which may indicate the significance of the C terminus in ICER localization. The wildtypes show a strong signal but do not show specific ICER localization.
Figure 4. **Micrographs of the Control, K0-52, K0-59, and K0-70.** These mutants are located most closely to the N terminus of ICER. We hypothesize that they may have decreased significance in the ubiquitination process due to their location. However, the strong ICER localization in the nucleus should be noted.
Figure 5. Micrographs of the Control, KO-71, KO-72, and KO-76. This control is one of the few exceptions where there is a noticeable ICER signal and it is only seen in the nucleus. As the position of the lysines approach the DNA binding domain, it becomes more apparent that their ICER localization is more strongly nuclear than otherwise. This would be consistent with the idea that the residues susceptible to ubiquitination in this domain are more relevant in ICER processing.
Figure 6. Micrographs of the Control, K0-87, K0-90, and K0-97. As the mutated residues approach the C terminal end, the effect on ICER localization is consistent with the previous cells in Figure 5. ICER is predominantly in the nucleus when it would otherwise not be, as seen in the control.
Figure 7. Micrographs of the Control, K0-100, K0-106, and NK0. K0-100 and K0-106 most closely resemble the previous mutants with nearby lysine mutations. Of note, the NK0 mutant can be compared to both the wildtype populations seen previously. The ICER distribution here is strong and even distributed throughout the cells.
Western Blot Analysis

In an effort to provide evidence in support of the immunocytochemistry results, Western blots were performed to qualitatively corroborate the significant nuclear localization bias of our mutant ICERs. It was expected that the controls, CK0 and NK0, would have the strongest ICER signal in the nucleus compared to the control and the wildtypes. The results show they do appear to have the strongest ICER expression indicated by the intensity of their bands, the complete knockouts also have the highest concentration of ICER in the nucleus when compared to the other mutants but not the wildtypes (Figure 8A). The evidence also confirms the presence of degraded forms of ICER in the complete knockouts, which is shown by the less intense bands further south on the gel. Concentrations of these degraded proteins are similar to that of the Cwt.

Interestingly, K0-59 has no ICER signal in either extract. This is inconsistent with earlier findings from the immunocytochemistry that showed a distributed pattern between the two. There is no instance where ICER is completely either in the nucleus or the cytosol. The extent of the difference between the signals is what is significant here. K0-90 and K0-97 are clear examples of the mutants ICERs showing strong nuclear localization. Overall, the bands representing the cytosolic ICER in the mutants are definitively less intense than the wildtype and the bands representing the nuclear ICER are diminished as well but contain a larger proportion of the overall distribution. This supports the hypothesis that the mutants ICERS remain in the nucleus for longer periods of time when compared to the wildtype.
Figure 8. Nuclear and Cytosolic Western Blots of ICER Mutants. A) The majority of the mutant ICERs have strong nuclear (N):cytosolic(C) ratios. Intensity of the bands representing the nucleus appears to be either as strong as or stronger than the cytosolic bands. B) The beta-actin bands are loading controls and show consistent protein concentration between samples.
Dual Luciferase Reporter Assay

Perhaps more important than the location of ICER is the efficiency at which it binds to DNA and represses gene expression. The ratio of luminescence in this assay is the indicator of the efficiency of each of the mutant ICERs. If the ratio is high, ICER is repressing less. If the ratio is lower, ICER is repressing more strongly. The data (Figure 9) indicates that the mutant ICERs are repressing genes more strongly than their wildtype counterparts. The wildtype cells had an average ratio of luminescence of about 0.07 while every mutant tested was less than 0.02. This is a significant difference in efficiency that may be attributed to the inability of the cells to remove ICER from the nucleus. The previous data support the assertion that the levels of ICER are elevated in the nucleus of these mutants and the luciferase assay seems to suggest that they are more efficient in their role as a repressor.

Figure 9. Dual Luciferase Assay Graphical Representation. The data show a significant difference between wildtype ICER efficiency versus the mutants.
Terminal Deoxynucleotidyl Transferase (TdT) dUTP Nick-End Labeling Assay

The goal of the TUNEL assay is to show levels of apoptosis in a given cell population. Specifically, we are interested in determining if there is a connection between rescuing ICER levels and increased apoptosis in melanoma cells. The data presented for the TUNEL assay (Figures 10-13) are preliminary and do not currently support our hypothesis that stronger nuclear localization of ICER may increase levels of apoptosis in melanoma cells. A positive result for apoptosis would look similar to what is seen in the control micrograph utilizing DNase I (Figure 10). The coloration is obvious and easy to see. However, looking at the micrographs for the mutants, it would seem as if there is a negligible effect on the viability of the cells. Out of the hundreds of cells visible using DAPI stain, there is no significant data for most mutants. On the other hand, KO-76 and KO-87 (Figure 12) have an appreciable signal compared to the rest. Though the signal is not as strongly represented in the micrographs as it is in the control, the light green glow may indicate the process of apoptosis on some diminished level. This may be evidence for further investigating the DNA binding domain. There are only few instances where the bright green intensity of the control was seen in the mutant populations. For example, KO-71 and KO-76 (Figures 11-12) have nine and three spots of significance, respectively. The mutant ICERs’ effect on apoptosis requires further research.
Figure 10. TUNEL Micrographs of the Control, Nwt, Cwt, and pCMV Renilla.
Figure 11. TUNEL Micrographs of K0-52, K0-59, K0-70, and K0-71.
Figure 12. TUNEL Micrographs of K0-76, K0-87, K0-90, and K0-97.
Figure 13. TUNEL Micrographs of K0-100, K0-106, and NK0.
**Discussion**

*ICER Lysine Mutants are Strongly Localized to the Nucleus of SK-MEL-24 Cells*

Due to its nature as a transcription factor, it is imperative that ICER remains in the nucleus of cells in order to provide for its function. Since it has been shown numerous times that ICER is marked for ubiquitin-mediated degradation and ICER levels are either extremely diminished or absent altogether in cancer cells, we have hypothesized that rescuing this protein may have a lethal effect on these cells. We have shown that combatting the expulsion of ICER from the nucleus by altering the sites at which it is marked for destruction is a viable first step in a path to potentially taking this research a step further and developing an ICER based cancer treatment.

Our results for immunocytochemistry are convincing enough to conclude that our ICER mutants are resisting ubiquitination. It is interesting that in the majority of conditions, there always seems to be a small amount of ICER in the cytoplasm regardless of the levels in the nucleus. This is indicated by the pinkish signal seen in all the mutants and even the controls. The evidence still strongly points toward the DNA binding domain of ICER being the area of most promise. For example, K0-87 and K0-97 (Figure 6) have the largest ratio of nuclear to cytosolic ICER. There is almost no signal originating in the cytosol. Future research in this area, specifically in this domain, may further elucidate the mechanisms involved. Unexpectedly, the NK0 and the CK0 (Figures 3 and 7) cells have ICER in their cytosol. These cells are complete lysine knockouts and if ICER degradation based on ubiquitination of the lysine residues was the
only mode of protein tagging taking place, it is unlikely there would be much, if any, ICER present in the cytoplasm. This seems to suggest that some other mechanism is either occurring simultaneously and compensating for the diminished efficiency of ubiquitination. Many such mechanisms do exist in nature and work in ways similar to ubiquitination.\textsuperscript{16} Examples of two such mechanisms are sumoylation and neddylation.\textsuperscript{17-18} These processes utilizing the proteins SUMO and NEDD8, respectively, are in a class called "ubiquitin-like proteins" and also modify cellular proteins post-translation, even for destruction, similarly to ubiquitination.\textsuperscript{19} It is possible that these mechanisms, or the others that exist, may have contributed to the unexplained levels of ICER in the cytosol.

\textit{Western Blot Analysis Confirms Higher ICER Concentration in the Nucleus than Cytosol}

The results from the Western blots are consistent with the conclusions illustrated from the immunocytochemistry. Supporting prior evidence, the results show a clear ICER localization to the nucleus. When considering previous work that has shown the absence of ICER in the nucleus of cancer cells, it is interesting to note the nucleus heavy bias in ICER concentration.\textsuperscript{20} Most of the mutant ICERs tested had nuclear:cytosolic ratios on par with that of the control or a more intense ratio favoring the nucleus (\textbf{Figure 8}). This provides further evidence to suggest that altering the lysine residues on the protein make it more difficult for the cellular machinery to degrade ICER. Due to beta-actin’s nature as a cytosolic protein, its strong presence in the nucleus seems to indicate cross contamination between the cellular fractions; although under specific cellular conditions, beta actin may be present in the nucleus.\textsuperscript{21} If this were the case, ICER concentration in the nucleus may be more diluted in our results than what they actually are. In terms of the
application of this information, ICER has already been shown to reverse tumor growth in rat prostate cancer.22 Establishing the nuclear localization of ICER in human melanoma cells is the first step on the way to testing the proteins effect on the viability of the cells and hopefully showing that ICER can cause apoptosis in these cells.

ICER Mutants Repress More Strongly than Wildtype ICER

ICER localization in the nucleus is not enough for the mutants to outperform the wildtype. In order for this to occur, the mutants must bind with their canonical CRE sequence in similar or greater levels. If the mutants were mainly located in the nucleus but did not bind to DNA efficiently, their presence would be redundant. The dual luciferase assay exhibits the strong repressing power of our mutant ICERs. The controls for this experiment indicated by the large bars (Figure 9) show what would be considered wildtype levels of ICER efficiency in cells that are already known to be ICER-deficient. The data for the mutants support the assertion that the mutants ICERs are much better at repressing genes that are transcriptionally affected by ICER. The ratio between firefly luciferase:Renilla luciferase is large and provides evidence for strong repression and results in the small bars in comparison to the wildtype controls. The conclusion drawn from this experiment is that the ICER mutants are much better repressors than the wildtype ICER.

As the TUNEL data presented here are preliminary (Figures 10-13), they do not currently paint a picture supporting the hypothesis that mutant ICERs will cause apoptosis in human melanoma cells on their own. A positive, apoptotic result would have shown a vast number of bright green cells in relation to the total number of cells present
indicated by the DAPI stain. The current data show no real correlation between increased mutant ICER localization in the nucleus and the viability of the cells. The lack of any appreciable results from this experiment suggests either a scientific dead end or human error resulting in misleading data. In either case, more research is needed to further investigate the potential link between our mutant ICERs and apoptosis in cancer cells.
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