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Effects of Cucurbitacins E, D and I on the Gene Expressions of Apoptotic, Autophagic and AKT-Mtor Pathways in SW480 Colorectal Cancer Cells

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Abstract

Backgorund: Many studies have reported the anticancer effects of cucurbitacins. However, related molecular events

opposite can hold true. In contrast, a high level of expression of pro-apoptotic BAX can correlate with poor results. Studies demonstrated that high levels of BAX can be associated with decreased survival and increased risk of relapse in all kinds of cancers [17-22]. As we described above, ndings challenge the dogma and suggest this viewpoint that anti/pro-apoptotic factors can be served in an unusual direction to inhibit cancer.

e mutant TP53 is in approximately 50% of human cancers. e modi ed TP53 loses its tumor-suppressive function and obtain new oncogenic activities whether through transcriptional e ects on various genes or by protein-protein interactions. us, it turns into an active antithetical protein having its own "social network" of interacting proteins and transcriptional targets which endows it with a gain of function (GOF) activities. Tumor cells gain resistance to cell death and become chemoresistance by recruiting the mutant TP53 interacting with proteins such as caspase 3, P300, P73, VDR, etc. [23,24]. SW480 and HT-29 are primary colorectal adenocarcinoma cell-lines with mutant TP53 [25], from which can bene t binding caspase-3 and inhibits its activation.

Autophagy is an intracellular degradation system that delivers cytoplasmic constituents to the lysosome [26]. Autophagy is activated in response to multiple stresses during cancer progressions, such as nutrient starvation, the unfolded protein response (ER stress), and hypoxia; besides, it is observed upon treatment of cancers with a broad spectrum of cytotoxic and targeted chemotherapeutic agents [27].

e mammalian kinase target of rapamycin (mTOR) is a primary regulator of the autophagic process and is regulated by starvation, growth factors, and cellular stressors [28]. Upstream of mTOR the AKT/PTEN pathway modulates mTOR activity. e interplay between the AKT/PTEN/mTOR pathway and the autophagic process is complex, and disruption of the molecular e ectors of the negative feedback loop of the AKT/PTEN/mTOR pathway may unbalance the e ects towards cell death with several outcomes [29].

In the extensive number of oncological researches, the isolation and puri cation of biologically active compounds from plants have been increased due to the discovery of potent antitumor drugs with high biodiversity and minimum side e ects [30]. We, therefore, set out to compare the cytotoxic e ects of the cucurbitacins E, D and I through measuring their IC50 and also by evaluating the expression of some prominent genes in death (apoptotic and autophagic) and survival (Akt/mTOR) pathways to infer (if feasible) the cause of the di erences in cytotoxicity e ects of these three types of cucurbitacins. e candidate genes were *BCL-2*, *BAX*, *p53*, *Aif* and *caspase-3* for apoptosis pathway, and *LC3*, *Beclin* and *ATG5* for autophagy as well as *Akt*, *mTOR* and *PTEN* for survival in the signaling pathway.

Materials and Methods

Reagents

Table 1: Nucleotide sequences of the primers used for real-time RT-PCR.

I respectively). Upon addition of cucurbitacins D, E and I, the cell viability was reduced to 78%, 61% and 62% at 10 µM of cucurbitacins until it reaches 61%, 20% and 25% at 50 µM, respectively. ere was a signi cant decrease ($p<0.01$) in the cell viability a er cucurbitacins E and I exposure, especially at high concentrations (≥80 µg/ml). e behavior of cucurbitacins E and I are similar, and at approximately 20 µM of cucurbitacins E and I cells reach to IC50; however, cucurbitacins D can only reduce the viability of the cells by 60 % (Figure 1).

Cytotoxic e ect of Cucurbitacin-E on HT-29 cells

Cells in the absence of cucurbitacins-E showed more than 90% viability. Cell viability was reduced to 50% at 6 µM, and it reached to 60%, at about 50 µM. ere was not a signi cant decrease in the cell viability at higher concentrations (≥30 µg/ml) (Figure 1).

E ect of Cucurbitacins on BCL2, BAX, p53, Caspase-3 and AIF mRNA expression in SW-480 cells

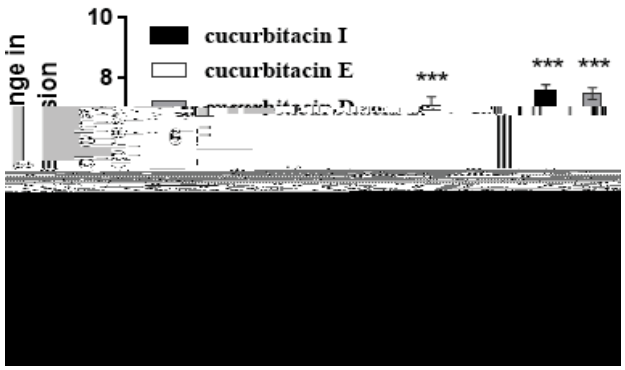
Upon addition of E and I cucurbitacins, p53 mRNA level was increased, but the e ect of cucurbitacin D on p53 mRNA expression levels was signi cantly ($p< 0.001$) more than that of cucurbitacins E and D with the approximately 7-fold increase in mRNA level. e expression of AIF mRNA in SW-480 cells was increased a er exposure to all types of cucurbitacins (Figure 2). e caspase-3 mRNA level of SW-480 cells was signi cantly increased ($p<0.001$) by cucurbitacin treatments in a concentration-dependent manner. Interestingly, the level of BAX mRNA was signi cantly decreased in SW-480 cells. By contrast, the expression of BCL-2 mRNA was signi cantly increased (Figure 3).

E ect of Cucurbitacin-E on BCL2, BAX, p53, Caspase-3 and AIF mRNA expression in HT-29 cells

We evaluated just apoptotic gene expressions for only HT-29 cells treated with cucurbitacin-E to have a comparative apoptotic gene analysis in a di erent primary cell line. Upon addition of cucurbitacins E, p53 mRNA was downregulated. e expression of AIF mRNA was also decreased a er exposure to cucurbitacin-E (Figure 4). e expression of caspase-3 was considerably increased ($p<0.05$), while that of BAX signi cantly dropped. Conversely, BCL-2 mRNA was signi cantly increased (Figure 4).

E ect of Cucurbitacins on ATG5, LC3 and Beclin-1 mRNA expression in SW-480 cells

A er the treatment of the SW-480 cells with cucurbitacins, the expression of LC3 and Beclin-1 mRNA was increased. Upon addition of cucurbitacin D and E, the expression of ATG5 mRNA increased, but



the e ect of cucurbitacin I on ATG5 mRNA expression was di erent with an approximately 3-fold decrease in mRNA levels (Figure 5).

E ect of Cucurbitacins on AKT, mTOR and PTEN mRNA expression in SW-480 cells

A er treatment of cells with cucurbitacins D and E, the mRNA expression of AKT and mTOR was increased, and the expression of PTEN mRNA was decreased. However, the expression pattern of AKT and PTEN was di erent a er the treatment of SW-480 cells with cucurbitacin I. Upon addition of cucurbitacin I, the expression of AKT and PTEN was increased and the expression of mTOR mRNA was decreased (Figure 6).

Discussion

MTT results demonstrated that the IC50 for cucurbitacins E, I and D in SW-480 were 20, 20 and 40 μM respectively. Cucurbitacins E and I had almost similar cytotoxicity e ect as a function of increasing their doses and also were more fatal to SW-480 CRC cells than cucurbitacin D. Unlikely, with increasing the dosage of cucurbitacin D, lethality went only up to 50% compared to 70% (cucurbitacin E) and 65% (cucurbitacin I). One reason why cucurbitacin D was less potent, would be the increase in the expression of AKT (1.9-fold) and mTOR (2.6 fold), and fall in the expression of PTEN (0.8 fold) which are in favor of the survival pathway.

Wild-type TP53 plays a signi cant role in suppressing tumorigenesis by inducing genomic stability, cell cycle arrest, or apoptosis while

mutant TP53 resists to cell death and apoptosis through binding and interacting with various proteins like caspase-3 [23,24]. P53 is mutant in SW-480 and the expression level of P53 (7 fold) in the cucurbitacin D treated cells was much more than E (expression levels was similar to control) and I (1.6 fold) treated cells, so this could be another explanation why cucurbitacin-D was less potent. us, it implicates that although cucurbitacin D treated cells induced caspase-3 gene expression 5 fold compared to control, this treated cells became resistant to cucurbitacin D through suppressing of caspase-3 by mutant p53. To study the cytotoxicity e ects of the cucurbitacins and the in uence of p53 in a di erent scenario, we exposed another colorectal primary cancer cell line HT-29 to cucurbitacin E, which similarly has mutant p53. we noticed that although the expression levels of caspase-3 in HT-29 was lower than that of SW-480 under the in uence of cucurbitacin E, it reached to IC50 in a lower dosage (6 µM), and interestingly, evaluation of the gene expression of p53 indicated that it was nearly suppressed with the 0.25 of expression level compared to non-treated HT-29 cells. us, the HT-29 cell line might miss the function of mutant p53 as a survival factor. Assessing the AKT-mTOR survival pathway demonstrated that cucurbitacin-I almost suppressed the expression of mTOR (just under 0.5), and AKT was expressed nearly in a similar quantity of control (1.28), while PTEN was upregulated 2.5-fold which overall is against the survival pathway. In cucurbitacin E, there was not any considerable tendency in favor or against of survival pathway compared to control.

Apoptosis pathway examination in SW-480 showed an unusual gene expression model. It was with the characteristics of upregulating BCL-2 and downregulating BAX, while caspase-3 and AIF were upregulated. As we explained earlier, the overexpression of BCL-2 and downregulation or suppression of BAX can be against cancer cell survival (9-22). e result for HT-29 cell line treated with cucurbitacin-E is in agreement with this idea, since even though the BCL-2 expression status was higher (8 fold), the IC50 was at a lower dosage (about 6 µM), and surprisingly the expression levels of caspase-3, on the other hand, was lower (3 fold) compared to the results of all three types of cucurbitacins in the SW-480 cell line. Our previous ow cytometry results demonstrated that puri ed cucurbitacins D, E and I induced apoptotic cell death in the human gastric cancer cell line (AGS). However, they showed a negligible e ect on the BAX mRNA level [31].

e autophagy gene expressions showed that cucurbitacin E and D treated cells in SW-480 upregulated the expression levels of LC-3, Becline-1 and ATG5 which is in favor of autophagic cell death. On the other hand, ATG5 was suppressed in cucurbitacin-I treated cells, and therefore autophagic cell death seemed unlikely to happen. is might be one reason that although caspase-3 expression was the highest in treatment with cucurbitacin-I, and conversely cucurbitacin-E had the lowest expression levels of caspase-3 (just over 4), there were no considerable di erences in their MTT results.

AIF is a mitochondrial protein, which can participate in caspase-independent apoptosis. AIF gene is a transcriptional target of p53 [32,33]. e e ect of cucurbitacin-E on the HT-29 cell line caused the suppression of p53 expression, and thus the AIF gene had no expression. Conversely, the p53 gene was expressed in all treatments of SW-480, which was followed by the expression of AIF. ese results illustrate that SW-480 capable of recruiting AIF to respond to caspase-independent apoptosis while HT-29 was not able to make it.

Conclusion

A better understanding of the mechanisms of BAX/BCL-2-independent cell death is crucial because various tumor cell lines have been shown to resist classical mitochondrial death pathways, as they lacking BAX or p53, or harboring mutations of these proteins which fail to respond to chemotherapeutic drugs and death ligands. Agents that overcome drug resistance in this type of cancer are of particular interest in drug development and cancer therapy. What is more, these results and other ndings challenge the viewpoint that a pro- or anti-apoptotic factor serves solely to inhibit or promote cancer, arguing instead that the factors in the apoptosis pathway have a dark side that can actually be served in their opposite direction. us, fundamental research on this unusual and speci c network of interactions could be promising in clinical settings. Cucurbitacins seem to have the potential to understand more about unusual interactions of apoptotic factors in cellular pathways and could also be more investigated for BAX/BAK independent apoptosis.

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Declaration of interest

This work was a thesis subject confrmed by the University of Tehran. The author(s) declare that there is no confict of interest. The authors alone are responsible for the content of the paper.

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