Clinical and Experimental Health Sciences

Hyaluronic Acid-Curcumin Complex Triggers Apoptotic Pathway in Breast Cancer Cells via CD44 Receptors

Ziba Mokhberi Oskouei[1], Gökhan Biçim[1,3], Ayşe Mine Yılmaz Göler[1,2], A. Süha Yalçın[1]

[1] Marmara University, School of Medicine and Institute of Health Sciences, Department of Medical Biochemistry, İstanbul, Türkiye
[2] Marmara University, Genetic and Metabolic Diseases Research Center, İstanbul, Türkiye
[3] Istanbul Kent University, Faculty of Dentistry, Department of Basic Sciences, İstanbul, Türkiye.

Correspondence Author: A. Süha Yalçın
E-mail: asyalcin@marmara.edu.tr

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ABSTRACT

Objective: Curcumin (CUR) was modified with hyaluronic acid (HA) to increase its water solubility and bioavailability. Our aim was to increase the uptake of CUR into the cells that express CD44 receptors and to compare the cellular effects in two different human breast carcinoma cells, MCF-7 and MDA-MB-231.

Methods: Hyaluronic acid-curcumin complex (HA-CUR) was synthesized and characterized. MCF-7 and MDA-MB-231 cells were grown under appropriate conditions and the effect of CUR and HA-CUR on cell viability was determined. Apoptosis levels of cells after treatment with CUR and HA-CUR were also measured. CD44 receptor levels of both cells were compared and then apoptosis levels were measured in MDA-MB-231 cells after saturation of CD 44 receptors with HA. In both cells expression of caspase-9 and PARP was analyzed to confirm apoptosis.

Results: In MCF-7 cells, the percentage apoptosis level of the CUR group was slightly lower than the HA-CUR group. In MDA-MB-231 cells, no statistically significant difference was found in the CUR group compared to the control group, but the apoptosis level of the HA-CUR group was higher than the control group. CD44 receptor levels were higher in MDA-MB-231 cells compared to MCF-7 cells. Blocking the CD44 receptors reversed the apoptotic effect of HA-CUR in MDA-MB-231 cells. Both CUR and HA-CUR had apoptotic effects in MCF-7 and MDA-MB-231 cells.

Conclusion: Conjugation of CUR with HA, which is specific for CD44 receptors, aids in its entry to target cells making it a powerful agent for targeted cancer therapy.

Keywords: Hyaluronic acid, Curcumin, MCF-7, MDA-MB-231, CD44 receptor.

1. INTRODUCTION

Curcumin (CUR) is a yellow pigment of the spice turmeric (*Curcuma longa*) which is chemically known as diferuloylmethane. It exhibits different pharmacological activities including those against oxidation, infection, atherosclerosis, and various cancers (1). At the molecular level, CUR has anti-inflammatory activity through the suppression of numerous cell-signaling pathways. However, properties such as lack of water solubility, poor stability, and low absorption rates have limited the clinical application of CUR (2).

Hyaluronic acid (HA), also known as hyaluronan, is a negatively charged water-soluble natural chain polysaccharide. It can interact specifically with CD44 receptors that are over-expressed in some cancer cells suggesting its use as an effective anti-cancer agent (3). HA has been used to modify CUR via covalent bonding and the amphiphilic polymer obtained by combining HA and CUR will self-assemble into nanoparticles in aqueous media (4). HA-specific receptor CD44 can be expressed on the surface of different cancer cells (5, 6).

In this study, we have modified CUR with HA in order to increase its water solubility and bioavailability. Our aim was to increase the uptake of CUR into the cells that express CD44 receptors and to compare the cellular effects in two different human breast carcinoma cells, MCF-7 and MDA-MB-231.

2. MATERIALS AND METHODS

Hyaluronic acid (HA) was purchased from Contipro Inc. (Dolni Dobrouc, Czech Republic). Curcumin (CUR, Sigma Cat. No.: C1386), 4-Dimethylaminopyridine (DMAP), N,N′-Dicyclohexylcarbodiimide (DCC), Dimethyl sulfoxide (DMSO) and Pefabloc were purchased from Sigma (Darmstadt, Germany). All reagents were used without further purification and were of HPLC or analytical grade. ApopNexin Annexin V-FITC apoptosis detection kit was from Merck

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Millipore (Darmstadt, Germany). Anti-PARP, anti-Caspase-9 and anti-IgG antibodies were purchased from Cell Signaling Technology (Boston, MA, USA).

2.1. Synthesis and Characterization of HA-CUR

HA-CUR was obtained as described by Manju and Sereenivasan (7). Figure 1 summarizes the process. Firstly, 80 mg of HA was dissolved in 8 mL of 1:1 H_2O/DMSO mixture and then 10 mg of DCC and 4 mg of DMAP were added. The solution was stirred for 1 h to activate the carboxylic group of HA. Then, 375 mg of CUR was dissolved in 5 mL of DMSO and slowly added to the above solution under nitrogen and the mixture was stirred well at 60–65°C for 6 h. The resultant solution was dialyzed using 3.500 Da MWCO dialysis kit (Sigma, PURX35005) against DMSO for 1 day and then against ultra-pure water for 3 days. Finally, HA-CUR was lyophilized and kept under refrigeration. Zeta potential, size distribution and polydispersity index (PDI) of HA-CUR were determined at 25°C using Zetasizer Nano ZS90 (Malvern Instruments, Worcestershire, UK). The concentration of CUR in HA-CUR was determined by spectrophotometric measurement. A stock solution of 1.0 mg/mL CUR was prepared in DMSO. From this solution, working standards were obtained by diluting 0.04, 0.08, 0.12, 0.24, 0.48, and 0.72 mL with 10 mL of DMSO. Maximum absorbance of CUR over the wavelength range 300-700 nm was observed at 440 nm. Absorbances measured at this wavelength were used to construct a calibration curve. Then 10 mg of HA-CUR was dissolved in 10 mL of PBS and its absorbance at 440 nm was used to calculate the CUR content.

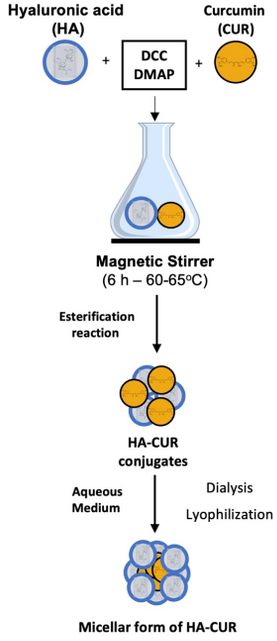


Figure 1. *Summary of the process used to obtain HA-CUR.*

2.2. Cell Culture

Breast cancer cell lines, MCF-7 (ATCC®; HTB22™) and MDA-MB-231 (ATCC®; HTB26™) were cultured in RPMI-1640 and minimum essential medium (DMEM), supplemented with 1% glutamine, penicillin (10,000 U/mL), streptomycin (10 mg/mL), and 10% fetal bovine serum (FBS). Cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2.

2.3. Measurement of Cell Viability

The stable tetrazolium salt of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was used to determine cell viability (8). MTT assay is a quantitative and sensitive method which measures the growth rate of cells. Cells were seeded into 96-well plates (6,500 cells/well) containing 100 µL of medium/well and incubated at 37°C to adhere. A medium containing 0.1% DMSO with cells was used as a control. The next day, CUR and HA-CUR were applied (0, 2.5, 5, 15, 20, 30, 40, 50 µg/mL). The effects on cell viability were determined at the end of 24 and 48 h by adding 10 µL of MTT solution. Cells were further incubated at 37°C for 3 h and their absorbance was measured at 570 nm. The absorbance is directly proportional to the number of viable cells.

2.4. CD44 Surface Receptors

The amount of CD44 receptors on the surface of MCF-7 and MDA-MB-231 cells was determined by indirect staining. Three tubes were prepared for each measurement and 200,000 cells/well were added to each tube. The first tube was used to locate the cells in the FSC-SSC panel and was not stained. The second tube was stained with the secondary antibody and used for non-specific binding. CD44 was determined by adding the first and second antibodies to the third tube. The tubes were incubated for 15 min at room temperature after adding 5 µL of the first antibody. Then 5 µL of FITC-labeled secondary antibody was added, the tubes were further incubated at room temperature in the dark for 10 min and analyzed by flow cytometry (BD Bioscience, FACSCalibur, CA, USA).

2.5. Detection of Apoptosis

ApopNexin FITC Apoptosis Detection Kit was used for detection of apoptosis after CUR and HA-CUR administration. MCF-7 and MDA-MB-231 cells were seeded in 6-well plates (200,000 cells/well), then CUR and/or HA-CUR (15 µg/mL) were added. After 48 h, cells were removed from the plates and suspended in 5 mL tubes containing the Annexin V binding buffer. Then 5 µL Annexin V and 10 µL PI solutions were added to 100 µL of cell suspension. Cells were gently vortexed and incubated for 15 min at room temperature in the dark. Finally, 400 µL of Annexin V binding buffer was added to each tube and flow cytometric analysis was performed. In each trial 10,000 cells were counted and run

in 3 replicates. Results were evaluated using the CellQuest program (Becton Dickinson, FACSCalibur, USA).

In MDA-MB-231 cells, apoptosis detection was also performed after saturating the CD44 receptors. For this purpose, cells were seeded in 6-well plates (200,000 cells/well) and left to adhere for 24 h. Then HA that was used for conjugation was added to saturate the CD44 receptors (10 µg/mL in fresh medium). The medium containing HA was removed after 24 h and refreshed with CUR and/or HA-CUR containing medium. Cells were further incubated for 48 h before detection of apoptosis as described above.

2.6. Western Blot Analysis

Western blot analysis of caspase-9 and poly (ADP-ribose) polymerase (PARP) expression was performed as described earlier in (8). Cells were seeded in 60 cm culture dishes ($2x10^5$ cells) and their protein was extracted with the lysis buffer. Protein concentration was determined with BCA Protein Assay kit (Pierce Chemical, USA) and approximately 40 µg of total protein was loaded to each well. Detection was performed using the West Pico chemiluminescent substrate kit (Thermo Scientific, USA) and the ChemiDoc MP System (Bio-Rad, USA).

2.7. Statistics

Statistical analysis was performed using GraphPad Prism 7.0 (San Diego, CA, USA). All results represent the mean ± SD of three independent experiments. Significance was tested using either a two-tailed Student's t-test or one-way ANOVA.

3. RESULTS

Maximum absorbance of curcumin was at 440 nm. Absorbances were measured at this wavelength and the standard curve of curcumin was obtained (y=0.001x + 0.0131, R^2=0.9994). Then 10 mg of HA-CUR was dissolved in 10 ml of PBS and its absorbance was measured. The amount of curcumin contained in HA-CUR was calculated using the standard curve and found to be half of the CUR added. The size distribution and zeta potential of HA-CUR were measured. The mean zeta potential, the mean intensity value and polydispersity index (PDI) were – 14.11 mV, 434.33 and 0.761 (n=3), respectively.

3.1. Cell Viability

MCF-7 and MDA MB-231 cells were grown as described in methods. The effect of CUR and HA-CUR on cell viability was investigated in a dose and time-dependent manner (Figure 2). In MCF-7 cells, at 15 µg/ml dosage and after 24 h, cell viability was 75.85% in the CUR treated group, while it was 68.77% in the HA-CUR group. After 48 h, these values were 94.07% and 91.94%, respectively. In MDA-MB-231 cells, at 15 µg/ml dosage and after 24 h, cell viability was 64.90% in the CUR treated group, while it was 17.83% in the HA-CUR group. After 48 h, these values were found to be 56.17% and 41.08%, respectively.

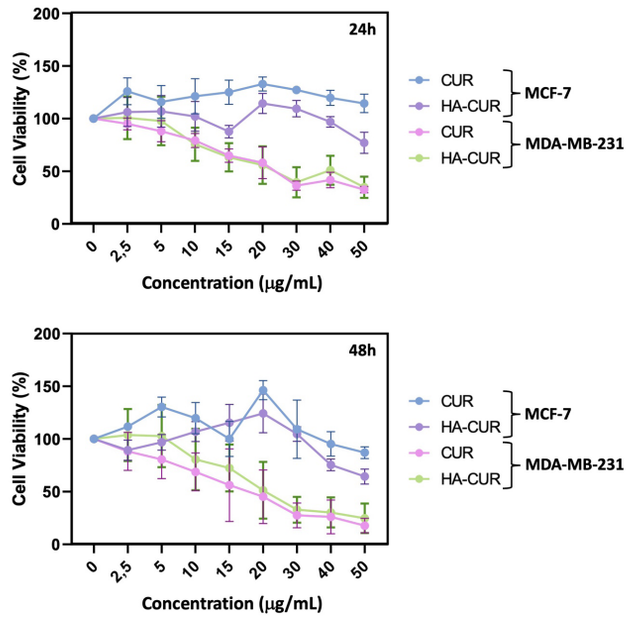


Figure 2. *Effect of CUR and HA-CUR on cell viability in MCF-7 and MDA-MB-231 cells: A) 24 h; B) 48 h. Values are expressed as the mean ± SD of three determinations.*

3.2. CD44 Receptor Levels of MCF-7 and MDA MB-231 Cells

MCF-7 and MDA MB-231 cells were stained with FITC-labeled anti-CD44 antibody and analyzed by flow cytometry (Figure 3). CD44 receptor levels were approximately 6 times higher in MDA-MB-231 cells (917.56%) compared to MCF-7 cells (152.52%).

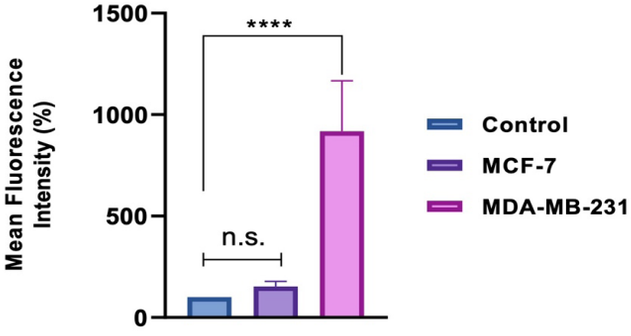


Figure 3. *CD44 surface receptor levels of MCF-7 and MDA-MB-231 cells. Values are expressed as mean ± SD of three determinations. ****p<0.0001. One-way ANOVA with Tukey multiple comparisons test was used.*

3.3. Effect of CUR and HA-CUR on Apoptosis of MCF-7 and MDA MB-231 Cells

The apoptotic effect of CUR and HA-CUR at 15 µg/ml dosage and after 48 h is shown in Figure 4. In MCF-7 cells, percentage apoptosis level of the CUR group was slightly lower than HA-CUR group (11.93 vs 17.63). There was no statistical difference between control and other groups. In MDA-MB-231 cells, no statistically significant difference was found in the CUR group compared to the control group (10.02 vs 14.27). The apoptosis level of the HA-CUR group was approximately 2.5-times higher than the control group (34.47 vs 14.27).

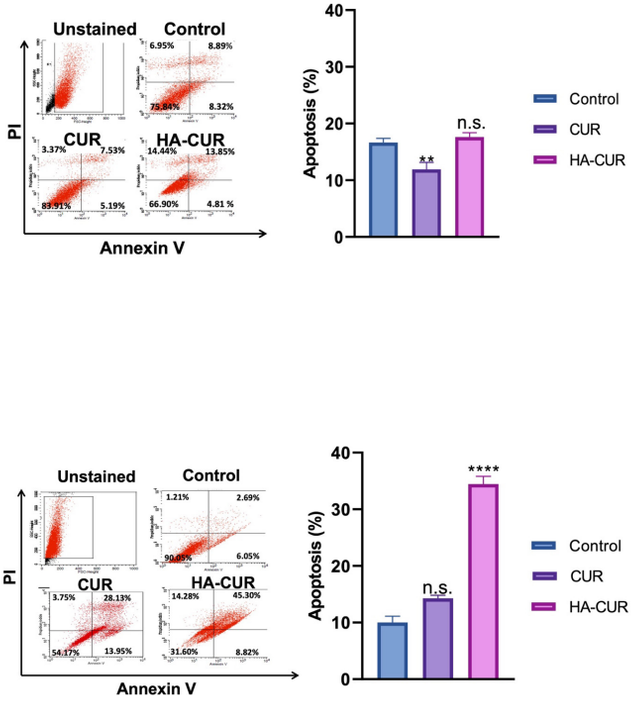


Figure 4. *Percentage apoptosis levels of A) MCF-7 cells and B) MDA-MB-231 cells after treatment with CUR and HA-CUR. Representative images and quantitative results of analysis are given. Cells were classified as live cells (Annexin V-, PI-), apoptotic cells (Annexin V+, PI – and Annexin +, PI+), and necrotic cells (Annexin V-, PI+). Values are expressed as mean ± SD of three determinations. **p<0.01; ****p<0.0001; n.s. p>0.05 vs control. One-way ANOVA with ordinary multiple comparisons test was used.*

3.4. Saturation of CD44 Receptors in MDA-MB-231 Cells

Figure 5 shows the results of CD44 receptor saturation in MDA-MB-231 cells. The percentage apoptosis of non-saturated control group (Control-NS) was 6.91% and that of saturated control group (Control-S) was 12.18%. There was a slight difference between these two groups (p=0.012). There was no statistically significant difference between the apoptosis levels of Control-S and HA-CUR-S (p=0.933). But apoptosis level of HA-CUR-S (24.07%) was significantly higher (p=0.0052) than that of HA-CUR-NS (13.00%).

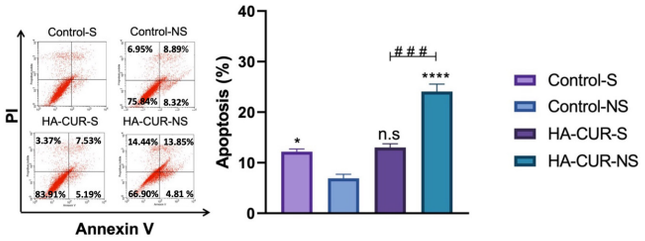
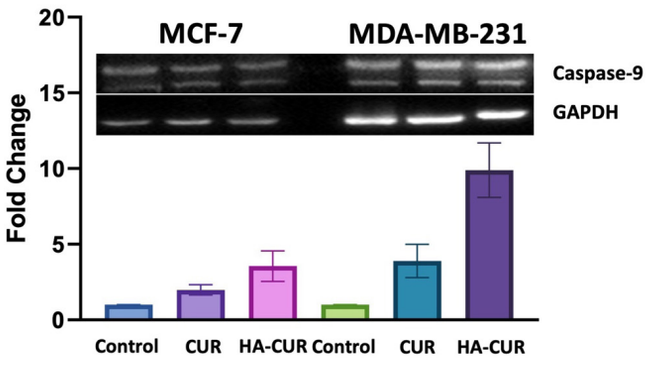


Figure 5. *Analysis of apoptosis in MDA-MB-231 cells before and after saturating the CD44 receptors.* Positive control cells were incubated with HA whereas negative control cells were not. *p<0.05; *****p<0.0001; vs control-NS*
n.s. p>0.05 vs control. ### p<0.001; HA-CUR-NS vs HA-CUR-S. One-way ANOVA with TUKEY multiple comparisons test was used.

3.5. Western Blot Assay

The results of Western blot analysis are shown in Figure 6. Cleavage of caspase-9 confirmed apoptosis in both cancer cell lines. Caspase-9 was cleaved from 35 kDa to 37 kDa when the cells were exposed to CUR. In MCF-7 cells, caspase-9 expression was increased 1.98 fold in the CUR group compared to control group. It was also increased 3.55 fold in the HA-CUR group. In MDA-MB-231 cells, the fold values of the same groups were determined as 3.88 and 9.88, respectively. Treatment with CUR upregulated cleaved caspase-9 in both cell lines compared to controls, confirming our apoptosis results. Up-regulation was approximately 3 times higher than MCF-7 cells in MDA-MB-231 cells. PARP expression was 0.324 fold lower than the control group in MCF-7 cells with HA-CUR application. PARP expression was 1.38 fold higher in the CUR group while it was 2.49 fold higher in the HA-CUR group compared to the control group in MDA-MB-231 cells.



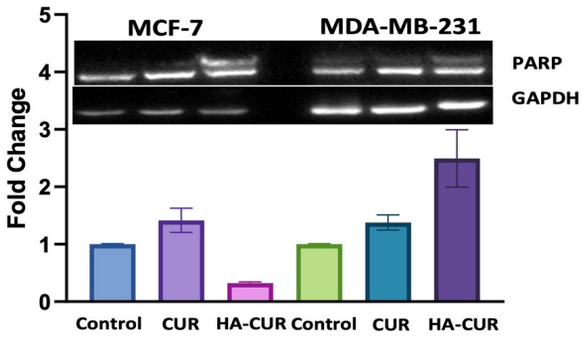


Figure 6. *Western blot analysis of caspase-9 and PARP protein expression after CUR and HA-CUR treatment in MCF-7 and MDA-MB-231 cells.* Band intensity was analyzed by densitometry. Fold changes of protein expression levels were calculated after bands were normalized to GAPDH.

4. DISCUSSION

Curcumin has been widely investigated as a drug candidate against various cancers. However, the molecule has poor bioavailability and stability in aqueous media at neutral pH values. In this study, we have conjugated CUR with HA to improve its water solubility and bioavailability. HA was chosen considering its affinity towards the cell-specific surface marker CD44. The conjugate (HA-CUR) was found to carry a net negative charge showing that HA molecules conveniently covered CUR. HA-CUR also formed micelles in aqueous media due to its amphiphilic character. Particle size distribution and polydispersity index determined by light scattering were found to be compatible with previous reports (7).

Anti-proliferative, anti-angiogenesis and anti-metastatic effects of CUR on cancer cells are gained by targeting signaling molecules such as growth factors, cytokines, transcription factors and genes modulating cellular proliferation and apoptosis (9). Hu et al. (10) reported that CUR is more active on breast cancer cells that are ER+, such as MCF-7 with regards to the ER – cells, such as MDA-MB-231. CUR induces apoptosis by regulating the expression of apoptosis related proteins in cancer cells (11, 12). In our study, the effect of CUR and HA-CUR on cell viability was determined in MCF-7 and MDA-MB-231 cells. In previous studies it was observed that CUR decreased the viability of K562 cells after 48 h (13). Similar studies exist in the literature reporting changes after 48 h in MCF-7 and MDA-MB-231 and cells (14). Therefore, we decided to use 15 µg/ml dosage and 48 h to further determine the apoptotic effects of CUR and HA-CUR. In MCF-7 cells, percentage apoptosis level of the CUR group was slightly lower than HA-CUR group and there was no statistical difference between control and other groups. In MDA-MB-231 cells, no statistically significant difference was found in the CUR group compared to the control group but the apoptosis level of the HA-CUR group was 2.5-times higher than the control group.

Yang, et al. (15) suggested that the apoptotic effect of CUR would increase when it enters the cell by receptor-dependent endocytosis. We have determined that CD44 receptor levels were 6 times higher in MDA-MB-231 cells compared to MCF-7 cells. In a previous study (16), the effects of receptors on metastasis was evaluated by comparing luminal breast lines in terms of CD44 and CD24 receptor amounts. Similar to our results, CD44 receptor levels were higher in MDA-MB-231 cells compared to MCF-7 cells suggesting that the metastatic ability of the cells decreased via this receptor. In order to validate the role of CD44 receptors on the apoptotic effects of CUR and HA-CUR, we have first saturated the receptors on MDA-MB-231 cells with HA and apoptosis measurements were then repeated. We observed that blocking the CD44 receptors reversed the apoptotic effect, suggesting the increased apoptosis levels in MDA-MB-231cells with unblocked receptors were related to the facilitated entry of HA-CUR through receptor-mediated endocytosis. HA induced apoptosis in all groups where CD-44 receptors were blocked. This finding is in accordance with previous reports showing that saturating CD44 receptors with low molecular weight HA can induce apoptotic pathways (17, 18).

Western blot experiments were conducted to confirm our apoptosis results. When the cells were exposed to CUR, Caspase-9 was cleaved from 35 kDa to 37 kDa. Treatment with CUR up-regulated cleaved caspase-9 in both cell lines. Up-regulation was 3 times higher in MDA-MB-231 cells. Hu et al. (10) reported that CUR inhibits Akt/mTOR phosphorylation pathway and suppresses expression of BCL2, an anti-apoptotic protein, and induces expression of BAX, an apoptotic protein that cleaves caspase-3. CUR also elevates Bax and p21 and decreases Bcl2 and p53 and NF-κBp65 levels in MDA-MB-231 cells (19). Increasing Bax/Bcl-2 ratio also results in the inhibition of cancer cell proliferation (20). All of these changes are clearly related to increased apoptosis levels we have observed after CUR treatment.

5. CONCLUSION

We have observed that both CUR and HA-CUR induced apoptosis in breast cancer cells (MCF-7 and MDA-MB-231). The effect was more pronounced in MDA-MB-231 cells which also had higher levels of CD44 receptors. Covering CUR molecules with HA, which is specific for CD44 receptors, aids their entry to target cells. Also, conjugating CUR with HA makes the molecule much more stable, increasing its apoptotic effect.

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Research idea: ZMO, ASY
Design of the study: ZMO, AMYG, ASY
Acquisition of data for the study: ZMO, GB, AMYG
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