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Knockdown of HSF1 sensitizes resistant prostate cancer cell line to chemotherapy

Roya Yousefi [1], Mahboubeh Kabiri [1*], Fatemeh Koohkan[2], Mahshid Heidari [1], Sedigheh Asad [1], Symzar Hosseinzadeh [3], Mohammad Hassan Nazaran [4]

1. *Department of Biotechnology, College of Science, University of Tehran, Tehran, Iran*
2. *Stem Cell Technology Research Center, Tehran, Iran*
3. *Department of Tissue Engineering and Applied Cell Sciences, School of Advanced Technologies in Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran*
4. *Department of Research and Development, Sodour Ahrar Shargh Company, Tehran, Iran*

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ABSTRACT

The treatment of prostate cancer patients usually starts with androgen ablation and followed by chemotherapy; however, in some cases the tumor develops resistant phenotype. Combination therapy is currently regarded as a cornerstone in cancer therapy to overcome the drug resistance. Herein, we investigated the combinatory effect of Docetaxel and Trastuzumab with a novel nanomedicine, BCc1. Also, we knocked down the expression of Heat shock factor-1, HSF1, in resistant Prostate Cancer cell line 3, PC3, using RNA interference, RNAi, to sensitize the cancer cells to the drug treatment. We observed down-regulation of Erb-B2 Receptor Tyrosine Kinase 3, ERBB3, B-Cell Leukemia/Lymphoma 2, BCL2, and Heat Shock Protein 90, HSP90, in HSF1 knockdown PC3 cells. Knockdown of HSF1 made PC3 cells more susceptible to Docetaxel treatment. Additionally, BCc1 nanomedicine was tested on prostate cancer cell line PC3 for the first time. It resulted in reduced metabolic activity in these cells. We propose that a combination of the gene therapy and the chemotherapy gives more favorable results in the treatment of refractory prostate cancer.

Corresponding Information: *Mahboubeh Kabiri, Department of Biotechnology, College of Science, University of Tehran, Iran.*
E-mail: mkabiri@ut.ac.ir

Abbreviations

HSF1, Heat shock factor-1; PC3, Prostate Cancer cell line 3; BCL2, B-Cell Leukemia/Lymphoma 2; DMEM, Dulbecco modified eagle's medium; ERBB3, Erb-B2 Receptor Tyrosine Kinase 3; gRT-PCR, quantitative Real-Time Polymerase Chain Reaction; HEK293, Human Embryonic Kidney 293 Cell line; HER2, Human epidermal growth factor receptor type 2; HSP90, Heat Shock Protein 90; HSPs, Heat shock proteins; IC50, half maximal inhibitory concentration; MTT, 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PBS, phosphate-baffered saline; Pca, prostate cancer; RNAi, RNA interference; shRNA, small hairpin RNA; β2m, β2 microglobulin protein

Introduction

Prostate cancer (PCa) is among the most common cancers in men, accounting for 20% of new diagnosis in the United States. Despite the decrease in the mortality rate over the last decade, Pca is still the second cause of cancer death in men (1).

Pca is one of the most heterogeneous malignant tumors. Cancer cells are initially dependent on androgens for proliferation; thus the first line treatment is androgen ablation therapy (2).

In 20-40% of the patients, a more aggressive tumor reoccurs which invades the tissues surrounding the prostate gland. At this stage, combination therapy can induce a clinically stable state for 1.5-3 years (3). Although the absence of androgen and the heterogeneity of the tumor usually leads to a clonal selection of androgen independent cells which are more likely to metastasize and are more resistant to available treatments (4).

Several studies have investigated the transcription factors expressing differently in resistant cell lines of PCa (5,6). Among them, serum response factor has shown to be a critical factor and its knock down increases the sensitivity of PC3 cell line to Docetaxel (7). Heat shock factor 1 (HSF1) is also shown to be distinctively expressed in the resistant cells of PC3 (8). Direct targeting HSF1 with inhibitors lowered the proliferation of the prostate cancer cells and reduced the tumor progression in animal models (9). HSF1 functions as a transcription factor for Heat shock proteins (HSPs) family (10). It is well documented that elevated levels of HSPs decreases programmed cell death and senescence and allows overexpression of mutated oncogenes and further growth of the tumor (11). Additionally, HSF1 is a signal modulator with various regulatory roles in kinase signaling cascades and energy metabolism that leads to the development of polyploidy phenotype in cancer cells (12). For this reason, HSF1 inhibitors are currently under investigation for anti-cancer effects (10).

In the development of aggressive androgen-independent phenotype, some other oncogenes and growth factor receptors have been highlighted in different studies. Among them, the anti-apoptotic factor, BCL2, overrides the tumor response to initial therapeutic treatments. BCL2 suppresses the release of caspase-activating protein cytochrome C from the mitochondria and therefore blocks the apoptosis (13). Although its basal expression in the epithelial cells of the prostate is low, it has been shown that BCL2 level increases upon androgen ablation therapy and this leads to the development of a more resistant phenotype (14). Suppressing NF-κ B and subsequent down regulation of BCL2 is associated with less invasive and metastatic phenotype of prostate cancer in mice (15). Several studies have shown some increase in apoptosis rate after antisense-mediated down regulation of BCL2 (16,17).

Human epidermal growth factor receptor type 2 (HER2) is a key player in the development of androgen independency through the outlaw pathway. Upon androgen deprivation, HER2 causes higher cell survival and mobility and reduced apoptosis (18). Although rare cases of primary prostate cancers show overexpressed HER2, one study showed an increase in its expression level upon progression to the aggressive phase (19). Some in vitro studies and clinical trials, however, demonstrated little efficacy of Trastuzumab as a monoclonal antibody against HER2, although combinations with other anti-cancer drugs or potential drug candidates have revealed some toxicity effects in vitro (20,21).

Nanotechnology has enabled the design of nano-sized particles with remarkable characteristics for enhanced selectivity and controlled release of the drugs (22). Some of these nanoparticles with chelating properties have shown therapeutic effects on a range of conditions including multiple sclerosis, diabetes, and Parkinson's disease. In tumors, the leaky vasculature system allows higher delivery of the nano-drugs and therefore less cytotoxicity to the normal tissues (23). Hafizi et al. synthesized the nanoparticle BCc1 based on a novel nanochelating technology (24–26) and confirmed its high affinity to iron. By chelating the iron ions, BCc1 reduces the activity of the iron-dependent ribonucleotide reductase which is required for DNA synthesis and cell proliferation. The same group showed witnessed the toxicity of the BCc1 on MCF-7 cells of breast cancer with no significant effect on wild type cells.(27). In a mouse model of mammary gland tumor, the size and growth rate of the tumor was effectively reduced upon treatment with BCc1. When used in a combination with doxorubicin, a synergistic effect was revealed in which, 50 times lower dose of doxorubicin resulted in enhanced life span of tumor-bearing mice (28). The magnificent anti-cancer potential of BCc1 and its general mechanism of action, motivates testing the efficacy of this product on other type of the tumors.

In this study, we took advantage of the RNAi gene silencing approach to knock down the HSF1 gene in PC3 cells and we showed reduced expression of HSP90, BCL2, and ERBB3 upon knockdown of HSF1. Interestingly, knockdown cells became more susceptible to Docetaxel. We observed for the first time the toxic effect of BCc1 nanomedicine on control and knockdown PC3 cells, therefore, we suggest more investigation on the efficacy of this drug in other prostate cancer models as a new potential chemotherapy.

Materials and Methods

BCc1 Nanomedicine

BCc1 nanomedicine was synthesized in Sodour Ahrar Shargh Company based on a technology patented in the United States Patent and Trademark Office with the subject of "Chelate Compounds" (29). The synthesis protocol is thoroughly explained in the previous study (27).

Cell culture

The PC3 cell line was obtained from Avicenna research institute (Tehran, Iran). Cells were cultured in complete Dulbecco modified eagle's medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (Gibco, Invitrogen), 100 unit/ml penicillin, 100 mg/ml streptomycin (Gibco, Invitrogen;), and 2 mM L-glutamine (Gibco, Invitrogen). The media of cells were changed every 3 days and the cells were passaged upon full confluence.

Designing the silencing constructs

The target sequences in the mRNA were chosen using the open online tools such as Ambion (www.invitrogen. com), siDRM (sidrm.biolead.org), and Promega (www.promega.com). The selected sequences were examined for their uniqueness in the HSF1 mRNA and unwanted off-target effect on other mRNAs. The favorable sequence should cover all splice variants of HSF1 mRNA. Two sense and antisense oligonucleotides including BamHI and EcoRI sticky ends and two complementary stems (sense and antisense strands of shRNA) and a 7 mer loop were synthesized (Metabion, Martinsried). Sense and antisense oligonucleotides (Fig. 1) were hybridized in the hybridization buffer in 72ºC for 30 min.

Afterward, they were ligated into BamHI and EcoRI restriction sites of pSilencer neo2.1 (Ambion; Invitrogen, Carlsbad, California, USA; Cat No. 5764) and then transformed into the Top10 F0 strain of Escherichia coli using the calcium chloride transformation method. The cloning was confirmed by colony-PCR with M13F and M13R primers and restriction analysis. To test the efficacy of the shRNAs, the constructs were transfected to Human Embryonic Kidney 293 Cell line, HEK293 (30), (Stem Cell Technology Research Center, Tehran, Iran) using Lipofectamine 2000 (Invitrogen) with the protocol provided by the manufacturer. 72 h later, the cells were collected for RNA extraction. Cells transfected with an empty vector were used as a control.

RNA extraction and quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

RNA of the control and HSF1-knocked down PC3 cells were extracted by Trizol (Invitrogen) according to the manufacturer's protocol. cDNA was synthesized using Mu-MLV reverse transcriptase and oligo primers (Thermo Scientific). Gene expression and real time PCR quantification analysis was conducted using standard SYBR Green kit protocol (Fermentas). Data were normalized to the expression level of the housekeeping gene β2 Microglobulin Protein, β2m. ΔΔCT was calculated for relative mRNA expression levels.

Virus proliferation and transduction

Lentiviral packaging plasmids, pMD2.G and psPAX2 (Addgene plasmids, #12259, #12260) plus small hairpin RNA, shRNAconstructs were transfected to HEK293 cell line using calcium chloride (31). In the next 3 days, media containing the viral particles were collected every 12 hours and concentrated using ultracentrifugation (50,000 g, 2h). Concentrated viral particles (MOI 30, TU/cell) were added to PC3 cells and incubated for 2 days before changing the media.

Treatment regimen

To test the drug efficacy on PC3 cells, cells were seeded in a 96-well plate (6000 per well). 24 hours after seeding the cells, different concentrations of each drug were applied to the cells. In "one dose" treatment approach, cell survival was measured after 2 days. In the alternative "refreshed" approach, 2 days after drug supplementation, a new dose of the drug was applied by changing the old media and then cell survival was measured after 24 hours.

MTT proliferation assay

To test cell survival, one hundred microliters of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma-Aldrich) solution, prepared in phosphate-baffered saline, PBS (5 mg/ml; 100X) was added to each well (32). Cells were then incubated at 37°C. After 5 h of incubation, the MTT solution was removed and the deposit was dissolved in 2-propanol (Merck). Absorbance was read using a spectrophotometer (Biotek) in 570 nm.

Statistical analysis

The non-parametric Mann-Whitney U test was performed in all the experiments to evaluate the statistical significance of the change compared to the control samples.

Results

Knockdown of HSF1 reduces the expression of genes in several oncogenic pathways

Two sequences of shRNA were designed to knock down HSF1 gene (Table 1). Both of the shRNAs effectively down regulated the expression of HSF1 with a 99% decrease induced by shRNA-1 and about 82% decrease by shRNA-2 (Fig. 1-A) comparing to an empty vector control. So, shRNA-1 was chosen for the rest of the experiments.

Expression of some candidate genes from the signaling pathways proposedly responsible for the resistance phenotype of prostate cancer were measured upon knockdown of HSF1 (Fig. 1-B). HSP90, one of the main players of the heat shock response, was decreased to almost 40 percent (0.42 ± 0.05). BCL2 was slightly but significantly down-regulated (0.7 ± 0.15).

To assess the changes of human epidermal growth receptor pathways, expression of HER2 and ERBB3 were analyzed. We could not detect HER2 in mRNA level, however ERBB3 expression was slightly lowered (0.81 ± 0.12) in HSF1 knockdown (KD) PC3 cells.

Table 1. The sequences of two designed shRNAs for targeting HSF1

shRNA 1	AATCCGCAGGTTGTTCATAGTCAGAATCAAGAGTTCTGACTATGAACAACCTGCG
shRNA 2	AATTCGCCCAAGTACTTCAAGCACAATCAAGAGTTGTGCTTGAAGTACTTGGGCG

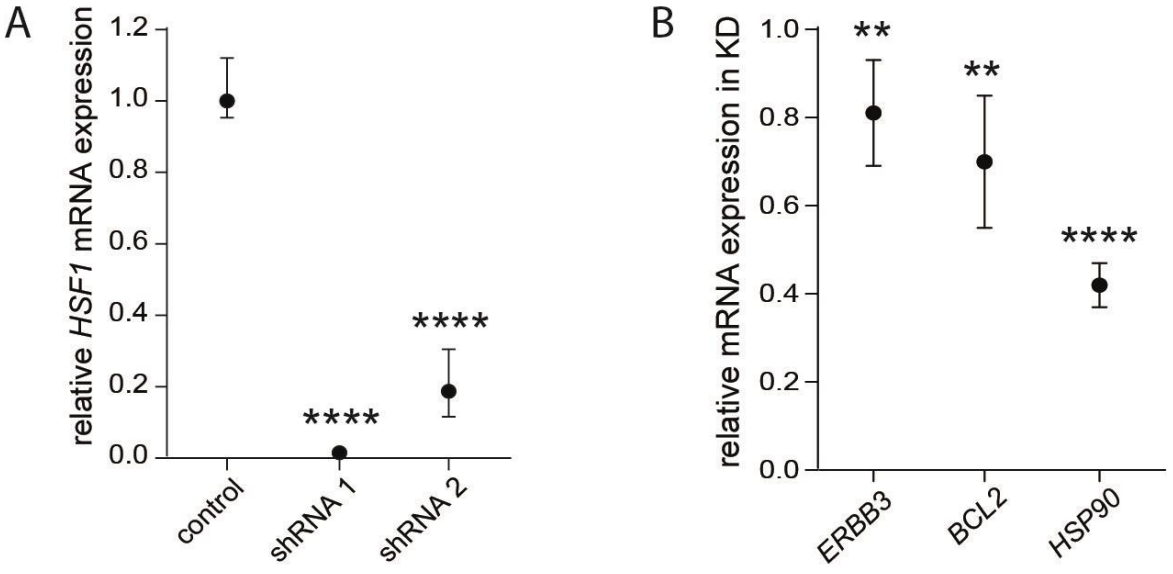

*Figure 1. Knock-down of HSF1 reduces the expression of genes in several oncogenic pathways. A. shRNA-1 and -2 induced 99 and 82 percent decrease in the expression of HSF1, respectively. β2m was used as the reference housekeeping gene. (Mean ± SEM, N = 3 independent experiments) B. change in the expression of selected genes upon knock-down of HSF1. (N = 3 independent experiments. Data are presented as mean ± SEM, ****p<0.0001, **p<0.01, Mann-Whitney U test)*

HSF1 knockdown PC3 cells are more susceptible to Docetaxel

One dose application of Trastuzumab caused no significant changes in metabolic activity of the control and knockdown PC3 cells (Fig. 2-A). Even when the drug was refreshed 48 h after the first dose, no significant change in the metabolic activity was resulted neither in the control, nor the knockdown cells (Fig. 2-B).

Similarly, one dose of Docetaxel was applied to the control and knockdown PC3 cells. No significant effect was detected (Fig. 2-C). With refreshing the drug 48 h after the first dose, however, the metabolic activity was dropped to 87, 86, and 74% of the control in response to Docetaxel concentrations of 5, 10, and 30 nM respectively (Fig. 2-D). Therefore, the decreased expression of HSF1 resulted in higher susceptibility to Docetaxel.

BCc1 nanomedicine inhibits the growth of PC3 cells independent of HSF1

BCc1, with a less understood molecular function, dropped the metabolic activity of both control and knockdown cells significantly.

Both control and knockdown cells showed similar response and a sigmoidal curve model was fit to the data points with a significant statistical score (R square higher than 0.9).When applied in one dose, the half maximal inhibitory concentration, IC50, was more than 1500 nM (Fig. 3-A) but refreshing the drug dose decreased the IC50 to around 1000 nM (Fig. 3-B).

Combination therapy does not enhance the toxicity on PC3 cells. We showed that Docetaxel inhibits the metabolic activity of the knockdown cells and BCc1 nanomedicine does the same to both control and knockdown cells. To test whether these drugs would show any additive effects, we applied different two-drug combinations on control and knockdown cells (Fig. 4). The concentrations were chosen based on the highest effect and the lowest applied dose. In most cases, the combined effect was not higher than the individual drugs. However, in knockdown cells, the combination of BCc1 and Docetaxel resulted in higher decrease (0.56) than each of them (Docetaxel 0.74, BCc1 0.78). Although, the reduction effect was not significantly higher than the sum of individual effects. Therefore, there was no synergic effect upon the use of drug combination.

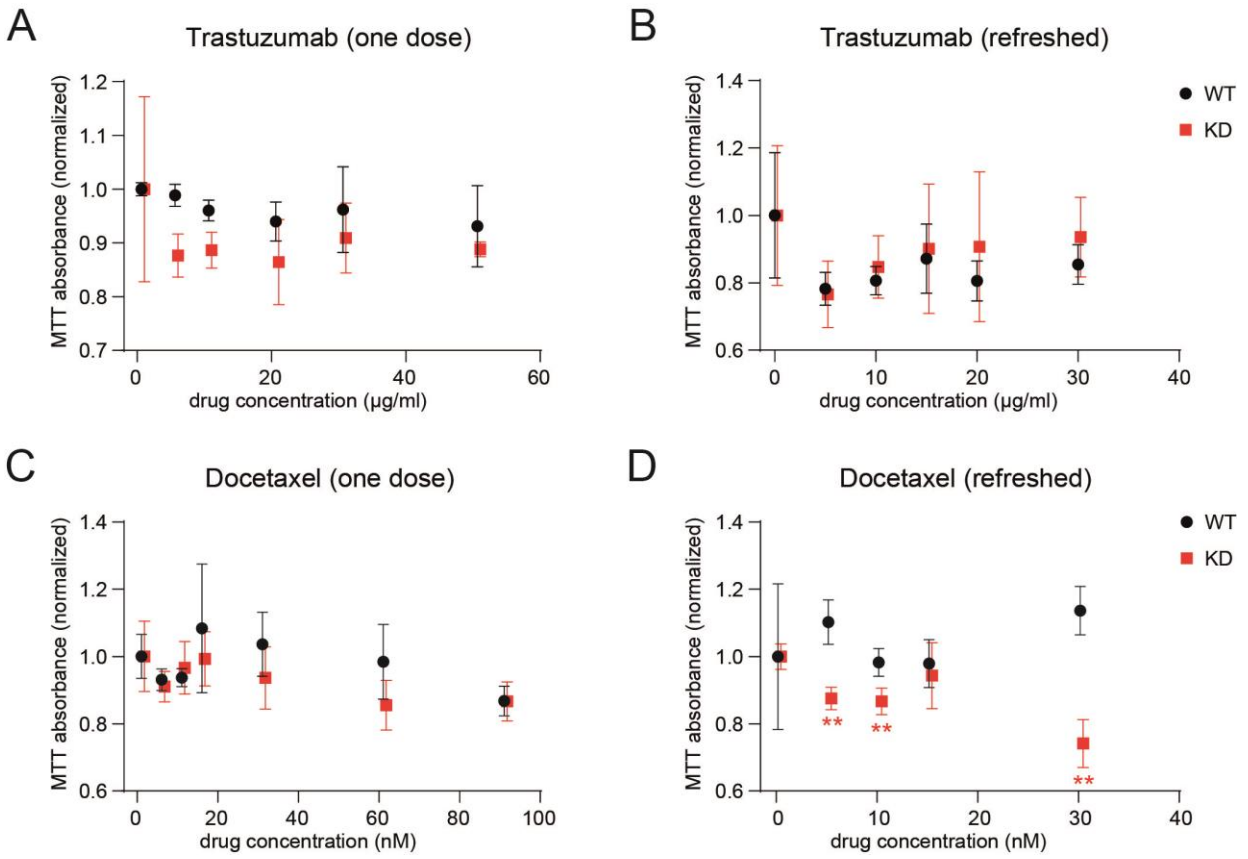

*Figure 2. Knockdown of HSF1 makes PC3 cells more susceptible to Docetaxel. Metabolic activity of the control and knockdown (KD) PC3 cells after A. one dose application of Trastuzumab, B. renewing the drug after 48 h C. one dose application of Docetaxel, and D. renewing the drug after 48 h. (Data shows the average values ± SEM in N = 5 independent experiments. MTT values were normalized to the respective no drug condition. *p<0.05, **p<0.01, Mann-Whitney U test)*

Discussion

RNAi as a therapeutic modality has attracted considerable attention in the last 10 years. As tumor formation and progression are almost always tied with changes in several key genes, silencing appropriate player of the team might cause considerable effect on cell proliferation, apoptosis, or sensitivity to anticancer agents. Different studies have reported the application of RNAi in cancer against oncogenes or antiapoptotic genes such as VEGF, p53, and BCL2 (33). Resistance to chemotherapeutic agents poses a major clinical concern in different types of cancers including PCa. This is why sensitization of cancer cells to chemotherapy is very important (34).

HSF1 is a necessary transcription factor which is required for acute response in proteotoxic stress conditions and it results in quick increase in transcription of downstream HSP genes (10). This pathway helps maintaining the global protein quality and allows cell survival in such stresses. Additionally, in many tumors, HSF1 is persistently active and is essential for multiple pathways of malignant transformation properties. Many studies have revealed a tremendous pleiotropy in the functions of HSF1 in cancer. Its expression has shown to be elevated in several tumors including metastatic prostate cancer (35). Transcription of heat shock protein 90 (HSP90), one of the key chaperones in heat and proteolytic stresses, is mainly under the regulation of HSF1. HSP90 and the other HSPs are also shown to play roles in some cancers including prostate cancer and some of their inhibitors are in clinical trials as drug candidates (36). In this study, we further proved that transcription of HSP90 gene is dependent on HSF1 as in KD PC3 cells, a 60% drop was measured in qPCR result of HSP90 gene. Moreover, for the first time we showed the knockdown of HSF1 leads to a decrease in the level of BCL2 expression. This finding is in agreement with Jakobs et. al. result on the effect of HSF1 expression on stabilization of BCL2 proteins through BAG3 induction that leads to the survival of colon cancer cells (37).

Previously the effect of Trastuzumab on different cell lines of PCa was reported and PC3 with the highest resistance against this drug was indicated (21). In this study, we followed the hypothesis that as HER2 is the main player in turning prostate cancer cells into androgen independent phenotype through outlaw pathway and because of HSF1 and its targets, HSPs, are shown to help outlaw pathway progression, silencing HSF1 could increase the sensitivity of PC3 cells to Trastuzumab as an HER2 inhibitor. Our results, however, did not support this hypothesis and although we showed a 20 percent drop in the expression of Errb3 (Her3), knockdown of HSF1 did not sensitize the cells to the treatment with Trastuzumab.

Similarly, we expected to see an increased effect by applying Docetaxel to HSF1-knockdown PC3 cells. This drug is being used as the main and the most effective chemotherapy in prostate cancer. In addition to its general function in all cancers (stabilizing microtubules), it is shown that in some cancers including PCa, it interferes with normal function of BCL2 as an anti-apoptotic protein; therefore, leads cancer cells toward apoptosis (38). Interestingly, HSF1 knockdown PC3 cells responded more strongly to Docetaxel treatment.

The third drug that its efficacy was tested in PCa cell line was BCc1 nanomedicine which its magnificent effects has shown in vitro and in vivo for several types of cancers and it is currently in clinical trial for gastric cancer(39). In this study, for the first time we tested the effectiveness of this nanomedicine on the most resistant cell type of PCa. Our results revealed the toxic effect of BCc1 on PC3 cells to be independent from HSF1 presence.

In current cancer therapy, combination of the drugs with different mechanisms is an approach to decrease the chance of resistance and side effects by lowering the applicable dose of the drugs. Many studies showed synergistic result of using two or more drugs. For example, BCc1 was shown to have synergistic effect when used in combination with doxorubicin to treat mammary gland tumors in mice (28).

With similar strategy, we tested the different combinations of the drugs considering their discrete mechanisms of action. Different two-drug combinations of Trastuzumab, Docetaxel, and BCc1, reduced the metabolic activity of the control and knockdown PC3 cells almost to the same levels the single drugs.

Conclusion

In conclusion, the knockdown of HSF1 in PC3 cell line, decreases the expression of Erbb3, Bcl-2, and HSP90 with important roles in the development of androgen-independent phenotype. For the first time, we showed that BCc1 nanomedicine has toxic effects on PC3 cells. PC3 cells were sensitized to Docetaxel due to the knockdown of HSF1. Therefore, we propose a combination of gene and chemotherapy for more favorable results in the treatment of prostate cancer.

Declaration

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Conflicts of interest/Competing interests

The authors declare no conflict of interest.

Authors' contributions

R.Y., M.K., F.K. Participated in study design, data collection and evaluation, drafting and statistical analysis.; S.A. and S.H. Contributed extensively in interpretation of the data and the conclusion. M.H.N developed and patented the nanodrug. M.H. Conducted drug tests. All authors performed editing and approving the final version of this paper for submission, also participated in the finalization of the manuscript and approved the final draft.

Ethics approval

Not applicable.

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