



BISBENZOXAZOLE DERIVATIVES HAD ANTI-PROLIFERATIVE EFFECT ON HUMAN CANCER CELLS

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ABSTRACT

A series of symmetric bis-benzoxazole derivatives were synthesized using one-pot cyclisation reaction between 4-fluoro substituted 2-aminophenol and suitable carboxylic acids. Synthesized compounds' anticancer activities were tested by using MTT assay on human prostate (DU145) and breast (MCF7) cancer cells. Screening results revealed that all compounds possessed a high level anti-cancer potential by significantly decreasing the cell proliferation in prostate and breast cancer cell lines. Our compounds exerted their anti-proliferative effects in a dose and time dependent manner. Our results suggest that they can be highly potent since they were biologically active even at low concentration ranges. Our study presents a series of new bis-benzoxazole based compounds with potential therapeutic effects against tumor cells. Therefore, characterization of new generation bis-benzoxazole derivatives will have a significant contribution on the development of new era anti-cancer drug candidates.

Keywords: Bisbenzoxazole, Breast cancer, Prostate cancer, Anti-proliferative, Anti-cancer

1. INTRODUCTION

One of the defining hallmarks of the cancer is the creation of abnormal cell masses that can rapidly proliferate and migrate into other tissues and can grow under different tissue environments [1-9]. Due to this abnormal cell proliferation and metastases patients suffer from organ failures and this situation eventually leads to death of the patients [10-12].

There are three main types of treatment options available against the cancer: surgery, radiation therapy, and chemotherapy [13-16]. Chemotherapy is the main focus of this study. Our goal was designing and later on examining new chemotherapeutic candidates against the most prevalent types of cancers. Chemotherapy is more effective when cancer is at its early stages [13-16]. Platinum based chemotherapeutics have been used in the field and recently new generation synthetics are developed and used as alternatives [13-18]. But despite of improvements, due to unwanted side effects of used chemotherapeutic drugs, new candidates are needed in the field [19-21].

These side effects can be listed as nausea, vomiting, diarrhea, constipation, mouth and throat sores, anemia and leucopenia due to decrease in the number of red and white blood cells and white blood cells respectively [19-21]. Chemotherapy can also affect the nervous system and produce certain symptoms such as tingling, burning, weakness or numbness in the hands and feet, soreness, tiredness, loss of balance and shaking or trembling [19-21].

In our study, we tested the anti-proliferative, therefore, anti-cancer effects of bisbenzoxazole based chemicals on prostate cancer cell line DU-145, breast cancer cell line MCF-7 and on Fibroblast cell line L929 as control. We focused on breast and prostate cancer cells due to their high incidence rates. Our reagents exerted anti-proliferative activity on these cell lines, therefore we are introducing two new synthetics that have chemotherapeutic potentials with possible side effects that should be further tested before their therapeutic applications.

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2. MATERIALS AND METHODS

2.1. CHEMISTRY

The synthesis method for the preparation of bisbenzoxazoles (RHE-247, RHE-238) are summarized in Scheme 1.

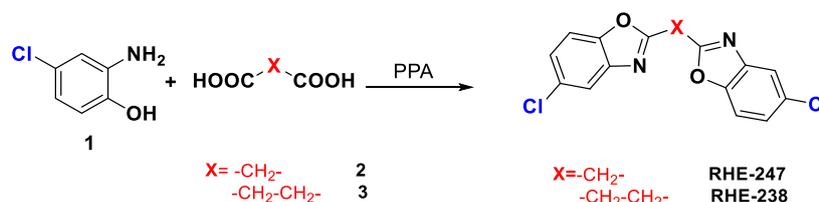
All reagents and solvents for the synthesis were of analytical and/or spectroscopic Agrade (Sigma-Aldrich and ACROS) and used without further purification. Each reaction was followed by thin layer chromatography (TLC) and purifications were performed with Flash column chromatography (Merck, 63–200 μm particle size, 60–230 mesh).

^1H NMR and ^{13}C NMR spectra (Bruker 400 spectrometer) and Fourier transform infrared (FT-IR) spectra (Perkin Elmer Spectrum One FT-IR spectrometer) were used to elucidate the structures of the products.

2.1.3 Synthesis

2.1.3.1. General Procedure for Synthesis of Bis-benzoxazole derivatives (Figure 1)

4-Chloro-2-aminophenol (**1**) (5 mmol) and the corresponding dicarboxylic acid derivatives (**2**, and **3**) (2.5 mmol) are refluxed for 13-15 hours after being dissolved in PPA heated in an oil-bath at 180 °C. The reactions were followed by TLC. After cooling, the reaction mixture was poured onto ice water and neutralized with 5N NaOH until it reached to a slightly basic pH (8–9) to make the precipitates. The resulting precipitate was filtered and washed off with cold water. Then compounds were purified by flash column chromatography and finally crystallized with a suitable solvent.



Scheme 1. General synthesis method of the compounds.

2.1.3.2 Bis(5-chlorobenzo[d]oxazol-2-yl)methane(RHE-247): The above procedure was followed with **1** and **2** to yield RHE-247 as a white crystalline solid (47% yield). The crystallization solvent was ethanol-water. **Rf** (Hexan:Ethylacetate 1:1)= 0,60 ; **mp**= 199 °C; **IR** (KBr, cm^{-1}) ν_{max} 3092, 2983, 1567, 1339, 860, 699, 677. ; ^1H NMR (400 MHz, CDCl_3) δ =7.64 (d, J =2.05 Hz, 2H, Ar-H), 7.40-7.37 (m, 2H, Ar-H), 7.26 (dd, J =2.05 Hz, J =8.68 Hz, 2H, Ar-H), 4.57 (s, 2H, $-\text{CH}_2$). ^{13}C NMR (100 MHz, CDCl_3) δ =160.8, 149.7, 142.2, 130.3, 125.8, 120.3, 111.5, 29.4.

1.3.3 1,2-Bis(5-chlorobenzo[d]oxazol-2-yl)ethane(RHE-238): The above procedure was followed with **1** and **3** to yield RHE-238 as a pink crystalline solid (41% yield). The crystallization solvent was ethanol-water. **Rf** (Hexan:Ethylacetate 1:1)= 0,51 ; **mp**= 179 °C; **IR** (KBr, cm^{-1}) ν_{max} 3090, 3066, 3048, 1562, 1344, 810, 777, 687. ; ^1H NMR (400 MHz, CDCl_3) δ =7.57 (d, J =2.06 Hz, 2H, Ar-H), 7.33 (bs, 1H, Ar-H), 7.22 (dd, J =2.06 Hz, J =8.64 Hz, 2H, Ar-H), 7.19 (bs, 1H, Ar-H), 3.49 (s, 4H, $-\text{CH}_2$). ^{13}C NMR (100 MHz, CDCl_3) δ =165.2, 148.5, 141.3, 128.9, 124.2, 118.9, 110.1, 24.4.

2.2. Biology

2.2.1. Reagents and chemicals

RHE238 and RHE247; 2 mg of RHE238 and RHE247 were dissolved in 1000 μl (1mL)of dimethyl sulfoxide (DMSO).

2.2.2. Cell culture

In this study the following cell lines were used: Fibroblast cell line; L929 from ATCC, breast cancer cell line; MCF7 from ATCC, and prostate cancer cell line; DU145 from ATCC. Cells were cultured in tissue culture plates with Roswell Park Memorial Institute media (RPMI 1640) media with %10 fetal bovine serum, %1 antibiotics (100 µg/ml penicillin and 100 µg/ml streptomycin) and sodium pyruvate. Cultures were incubated at 37°C in an atmosphere of 95% air and 5% CO₂.

2.2.3. Cell plating

Adherent cells from confluent cultures were detached. Cell counting was done by using Trypan Blue dye which stains the dead cells with dark blue color. The dye cannot penetrate through the live cells therefore we could differentiate between live and dead cells and have a reliable live cell number for the plating.

100.000 cells in 100µl were seeded in individual wells of 96 well tissue culture treated plates and allowed to adhere to the surface by overnight incubation at 37°C and 5% CO₂ before adding the reagents. 0,15 µM, 0,20 µM, and 0,30 µM of reagents were added into appropriate wells. Afterwards the samples were incubated at 37°C and 5% CO₂ humidified incubator for different time-points; 24h, 48h, and 72h.

2.2.4. Cytotoxicity evaluations:

MTT assay

Cell viability was evaluated by using MTT assay. This assay is based on the ability of viable cells to metabolize yellow tetrazolium salt MTT to purple formazan crystals by mitochondrial succinate dehydrogenase and spectrophotometric measurement of the product at 570nm.

Briefly, cells were seeded at a density of 1x10⁵ per well in 96-well plates; subsequently, after overnight incubation, they were treated with various concentrations (0,15 µM, 0,20 µM, and 0,30 µM) of RHE238 and RHE247. Cells were put back to 37°C 5% CO₂ incubator for 24 hours, 48 hours and 72 hours incubation. The untreated or DMSO treated well was considered as a negative control, and all samples were prepared in triplicates.

After 24h, 48h, and 72h of incubation, 10 µL of MTT reagent was added into each well and samples were further incubated for 4 h at 37°C, 5% CO₂. As a last step, 100µL of SDS based ROCHE Detergent reagent was added into each well. Cytotoxic effects were monitored by measuring the absorbance values of each well at 570 nm.

2.2.5. Statistical analysis

In order to determine the % cell viability average absorbance value of the reference blank sample was subtracted from each sample's average absorbance. The equation used for the calculations is given in the below and further plotting as well as statistical analysis were done by GraphPad Prism Software version 5. For each condition there were nine independent data points and unpaired two tail t-test was done to draw the statistical significance.

$$\% \text{ Viability} = \frac{\text{Sample absorbance} - \text{Reference absorbance}}{\text{Reference absorbance}} \times 100$$

3. RESULTS AND DISCUSSION

3.1. RESULTS

Both of our reagents had anti-proliferative effects on prostate (DU145) and breast (MCF7) cancer cells as well as fibroblast (L929) cells. This observed effect was dose and time dependent. We used

different concentrations of each tested chemicals (0,15 μM , 0,20 μM , and 0,30 μM) and cells were incubated for 24 hours, 48 hours and 72 hours.

With increasing concentrations of the reagents there was a more robust decrease in the percent cell viability of MCF7 breast cancer cells, DU145 prostate cancer cells and L929 fibroblast cells compared to their respective control groups (Figure 1-3). These differences were statistically significant. Longer incubation times lead to more substantial decreases in the percent cell viability compared to non-treated control groups (Figure 1-3). RHE 238 was more potent anti-proliferative reagent than RHE 247 at earlier time points (Figure 1-3). It exerted more substantial decrease at earlier time points with lower concentrations on all studied cell types compared to RHE 247 (Figure 1-3). Whereas, at later time points and higher concentrations of RHE 247 and RHE 238 anti-proliferative activities on breast cancer, prostate cancer and fibroblast cell lines were comparable.

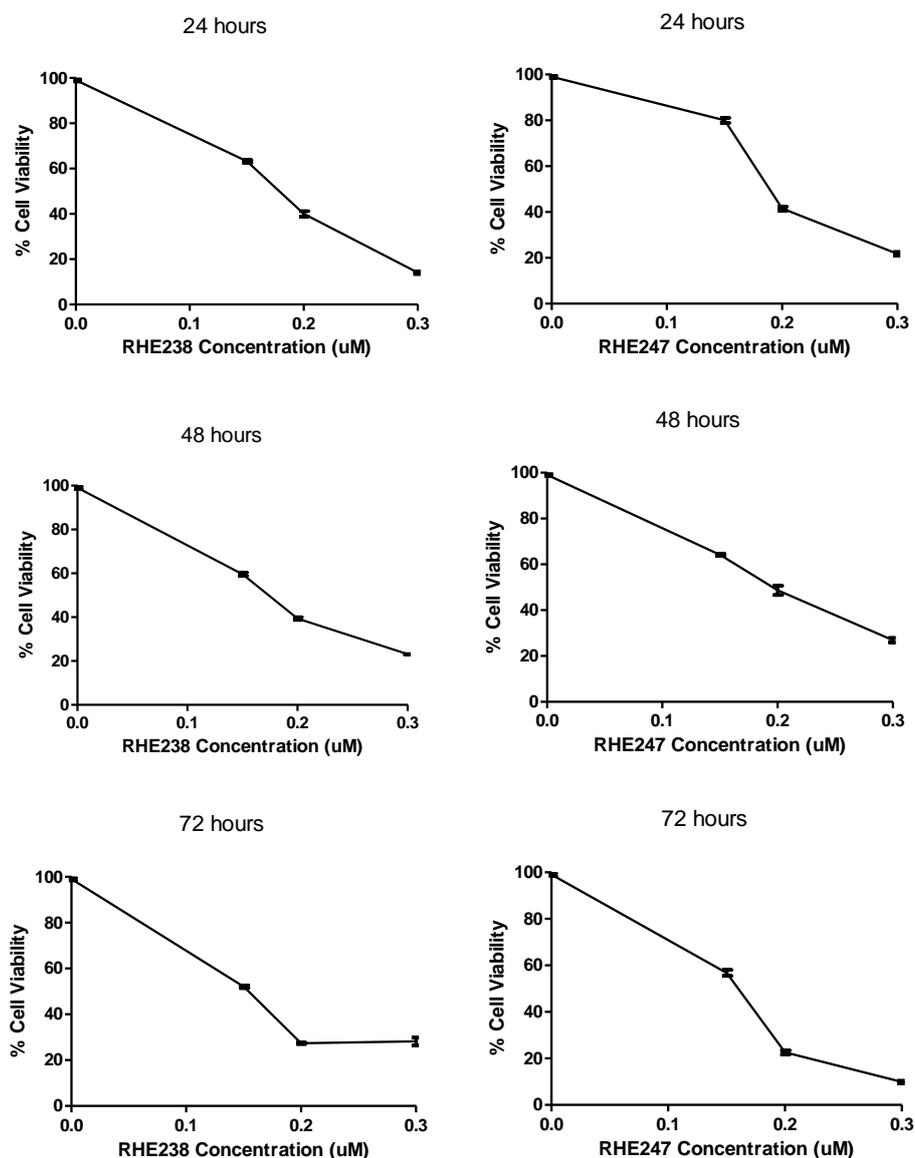


Figure 1. Anti-proliferative effect of RHE 238 and RHE 247 on human prostate cancer cell line DU145: DU145 cells were treated with RHE 238 & RHE 247, at different time points; 24h, 48h, 72h; and with different concentrations of the chemicals: 0,15 μM , 0,20 μM , and 0,30 μM . MTT assay was performed to measure the % cell viability. N=4.

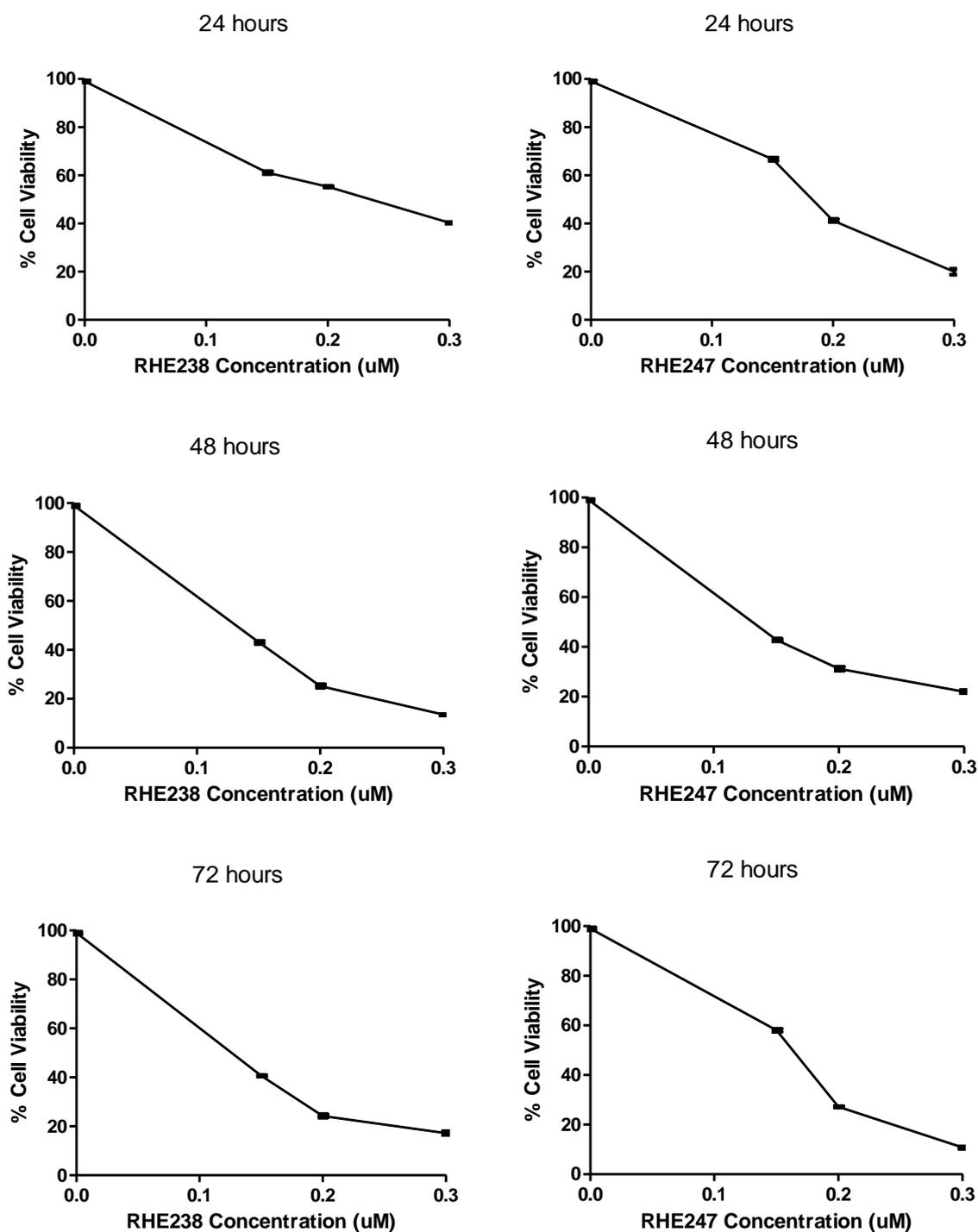


Figure 2. Anti-proliferative effect of RHE 238 and RHE 247 on human breast cancer cell line MCF7: MCF7 cells were treated with RHE 238 & RHE 247, at different time points; 24h, 48h, 72h; and with different concentrations of the chemicals: 0.15 μM, 0.20 μM, and 0.30 μM. MTT assay was performed to measure the % cell viability. N=4.

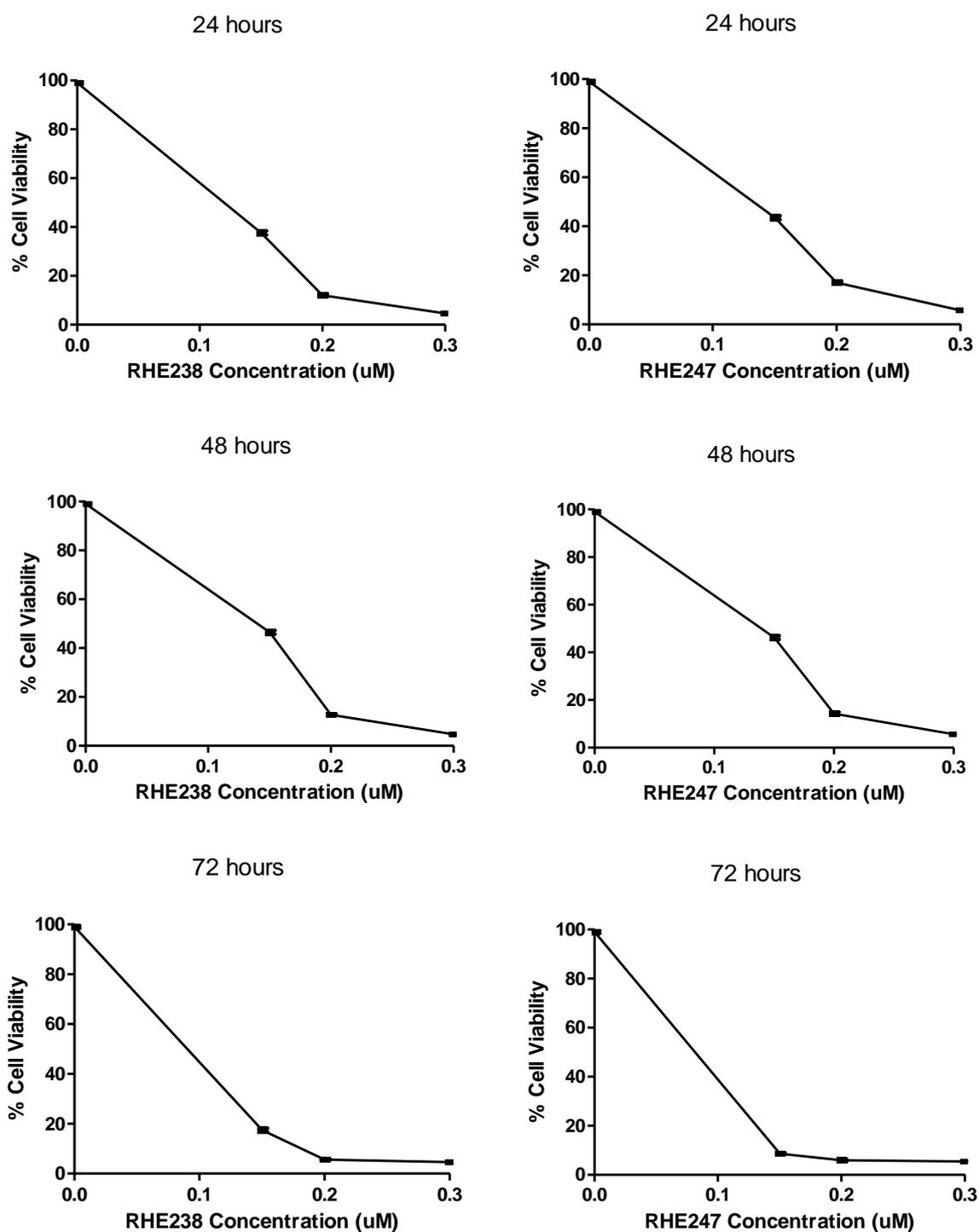


Figure 3. Anti-proliferative effect of RHE 238 and RHE 247 on mouse fibroblast cell line L929: L929 cells were treated with RHE 238 & RHE 247, at different time points; 24h, 48h, 72h; and with different concentrations of the chemicals: 0,15 μM , 0,20 μM , and 0,30 μM . MTT assay was performed to measure the % cell viability. N=4.

3.2. DISCUSSION

Cancer incidence rates have increased in the last decade and are expected to increase more since exposure to the risks factors of cancer have marked increase [22]. The conventional treatment ways of cancer (chemotherapy, surgery and radiotherapy) are not as effective as expected in some cases due to resistance mechanisms against the drugs as well as side effects of the treatments [19-21]. Chemotherapeutics can be divided based on their relationship with the other drugs, how they work, and their chemical structures [17, 18]. There are many drugs/ medicines that work through more than one way and can belong to more than one group [17, 18]. Chemotherapeutic drugs have dramatic adverse effects on normal cells, leading to disorders in their function [19-21]. Many of these side effects, such as general fatigue, anemia, loss of appetite and bleeding problems result after application of each dose to the patient, and some problems such as those related to the cardiovascular system or lung damage appear after completion of the treatment [19-21]. In some cases, resistance to the chemotherapeutics may develop and alternative drugs should be used to treat the cancer [17-21].

During the course of our research for the generation of the new bioactive benzoxazole compounds, we have synthesized a plethora of bisbenzoxazole derivatives and tested their anti-cancer activities by previous studies [23]. Benzoxazoles act as anti-cancer agents [24]. The active compounds are structurally unique bisbenzoxazoles in which the 2-position of one benzoxazole is joined to the 2-position of a second benzoxazole structure. In the current study, we tested the anti-cancer potential of a new set of bisbenzoxazole derivatives to further characterize them.

The objective of the study was assessing the anti-proliferative effects of the bisbenzoxazole based RHE 238 and 247 molecules on the breast cancer cells MCF-7, prostate cancer cells DU-145 and fibroblasts L929. MTT assay was used to determine the percent cell viability of our treated samples. Both of our reagents exerted anti-proliferative effect on breast and prostate cancer cells. This effect was dose dependent. Moreover, our reagents had anti-proliferative effect on the healthy cells as well. Their activity against fibroblasts underlines that they might also lead to unwanted side effects if their dosage is not well regulated during a possible treatment regimen.

4. CONCLUSION

The results of our study supports that, bisbenzoxazole derivatives RHE 238 and 247 molecules have anti-proliferative effect on prostate cancer cells DU-145, breast cancer cells MCF-7 and Fibroblast cells L929. Bisbenzoxazole based RHE 238 and 247 molecules are potent candidates to eliminate the tumor cells in the breast and prostate cancer patients but they might also lead to side effects due to their non-selectivity and anti-proliferative activities on fibroblasts. Therefore, it is crucial to adjust their dosage properly to prevent unwanted side effects during their possible applications in the future. Currently, we are evaluating their mechanism of action at molecular level in breast and prostate cancer cells.

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