The Effect of Newly Synthesized Heterosteroids on miRNA34a, 98, and 214 Expression Levels in MCF-7 Breast Cancer Cells

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Abstract Hybrid anticancer drugs have emerged as great therapeutic options that can effectively overcome most obstacles facing conventional anticancer drugs. miRNAs are considered as class of non-coding RNAs that can negatively regulate protein coding gene expression. miRNA expression is commonly altered in cancer cells. The current work aimed to test the effect of new pro-apoptotic heterosteroids on some drug resistance related miRNAs expression levels (miRNA34a, 98, and 214) in MCF-7 breast cancer cells. After cell treatment with these compounds **4**, **6**, **7**, **13**, **18**, **21**, **22** and **24**, miRNAs were extracted and subjected to reverse transcription and subsequent PCR amplification using Real Time-PCR technique. The expression levels of miR-34a, miR-98 and miR-214 were quantitatively determined. The study revealed that the expression levels of miR-34a, miR-98 and miR-214 were up-regulated upon treatment with tamoxifen, which was used as a positive control drug, as compared to control cells. Strikingly, the levels of miR-34a, miR-98 and miR-214 expression were significantly down-regulated when treated with most of the new heterosteroids as compared to control cells. These results could indicate the promising effects of these new heterosteroids on reducing drug resistance as compared to tamoxifen drug. As well established, cells develop drug resistance to tamoxifen.

Keywords Breast cancer · Steroids · Cytotoxicity · Apoptotic genes · miR-34a · miR-98 · miR-214

Introduction

Breast cancer is the second most common cancer type and the most prevalent among women [1]. Studies confirmed that alteration in different gene expressions is involved in breast cancer development [2]. Consequently, alterations in proliferation and apoptosis pathways have been used as targets for treatment [3]. Hybrid anti-cancer agents were recently used to diminish the toxicity and enhance specificity [4]. It is developed by the addition of heterocyclic rings to steroids. This combination leads to a change of their physiological activity and the appearance of new promising pharmacological and biological properties [5]. The change in the expression of certain genes dramatically affects the development and progression of breast cancers. This change could be mediated through many factors from which miRNAs are very potent regulators of gene expression in cells [6]. Many miRNAs were figured out as key players in cancer progression, metastasis, chemotherapeutic multidrug resistance and endocrine resistance in breast cancer [7]. Consequently, miRNAs have been shown to have both diagnostic and prognostic significance, in addition to, being important targets for cancer treatment [8, 9]. Drug resistance is considered as a major obstacle facing cancer chemotherapy and accounts for the failure of chemotherapy and finally mortality [10, 11]. Despite the well known role of miRNAs in cancer, the exact mechanism of miRNA role in drug resistance still not fully clarified. In normal cells, there is balance between pro-apoptotic and anti-apoptotic programs, however, in cancerous cells this balance is

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shifted toward cell survival [10]. miRNA-34a, miRNA-98 and miRNA-214 have a well established positive role in regulating apoptosis and reducing cell proliferation [12, 13]. Moreover, miRNA-34a has been established to be associated with drug resistance in cancer [14, 15]. However, less is known about the role played by miRNA-98 and miRNA-214 in modulating drug resistance. miRNA-34a is one of miRNA-34 family members which comprises three processed miRNAs that are encoded by two different genes. miRNA-34a is encoded by its own transcript while miRNA-34b and miRNA-34c share a common transcript. miRNA-98 is a member of let-7 family, which comprises 13 members (let7-a-1, a-2, a-3, b, c, d, e, f-1, f-2g, i, miR-98, and miR-202). Let-7 family members were found to target similar genes and play similar roles. Let-7 was found to negatively regulate other oncogenes and cell cycle regulators leading to cell cycle arrest by promoting the transition from G1 to S phase [16–19]. In breast cancer, miRNA-214 was found to be reduced, however, the functional significant relevance of this finding remains non-understood [20, 21]. miRNA-214 was reported to have a tumor suppressor effect [22]. The present study was conducted to test the effect of new promising heterosteroids on miRNA34a, 98, and 214 expression levels in MCF-7 breast cancer cells. The role played by miR-34a in drug resistance was tested. Moreover, we tried to investigate whether miR-98 and miR-214 have a similar role in drug resistance.

Materials and Methods

Cell Propagation, Maintenance and Treatment

Breast cancer MCF-7 cells were purchased from ATCC (American Type Culture Collection) and maintained in the proper conditions. The cells were cultured in Dulbecco's modified Eagle's Medium (DMEM) (Lonza, Belgium) supplemented with 10% fetal bovine serum (FBS), 4 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin sulfate at 37 °C in a humidified incubator with 5% CO₂. The cells harvested after trypsinization (0.025% trypsin and 0.02% EDTA) and washed twice with Dulbecco's phosphate-buffered saline (DPBS). When the cell density reached approximately 80%, cells were split for further culture. The experiments were made up when the cells were in the logarithmic growth phase. Breast cancer MCF-7 cells were seeded in 24 well plate at a density of 30,000 cells/well. The second day after seeding, cells were treated with compounds **4**, **6**, **7**, **13**, **18**, **21**, **22** and **24** at a concentration equivalent to IC₅₀ values. These compounds were synthesized and subjected to cytotoxicity analysis in a previous work [23]. The structures of these

compounds are illustrated in Fig. 1 and their IC₅₀ values are listed in Table 1.

Quantitation and QRT-PCR Measurement of miRNA-34a, miRNA-214 and miRNA-98

miRNA was extracted from total RNA using a miRNeasy kit (Qiagen). The miScript PCR system enables sensitive, specific miRNA quantification and profiling using SYBR Green real-time PCR. The miScript PCR system covers all the steps involved in conversion of RNA to cDNA and subsequent real-time PCR detection of miRNAs. MiRNA profiling of low-quantity RNA samples requires the following 3 steps: reverse transcription using the miScript II RT kit, preamplification using the miScript PreAMP PCR kit and miScript PreAMP primer mix and real-time PCR using the miScript SYBR Green PCR kit and specific primers for miRNA-34a, miRNA-214 and miRNA-98 that were provided from Qiagen. The cycling conditions were as follows: Denaturation for 15 s at 94 °C, Annealing for 30 s at 55 °C, Extension for 30 s at 70 °C. Then fluorescence data using MiniOpticon™ Bio-Rad Real Time Thermal Cycler Collection was performed.

Statistical Analysis

The data were analyzed using student *t* test to detect the significant difference among the studied compounds. All the data are expressed as Mean ± standard error mean. A level of *P* < 0.05 was defined as statistically significant.

Results and Discussion

Molecular Biology Assay

Expression Levels of miR-34a, miR-98 and miR-214

The mechanisms of resistance to chemotherapeutic agents are complex and not fully understood. Our previous study [23] revealed the promising effect of newly synthesized heterosteroids (**4**, **7**, **18**, **22**, **21** and **24**) on apoptotic pathway and cell cycles. This study figured out the promising effects of these compounds is mediated through their effects on CCND1, Survivin, BCL-2, CDC2, P21 and P53 genes. Compounds **4**, **7**, **18**, **24** down-regulated CCND1, Survivin, BCL-2 and CDC2 significantly, while compounds **21**, **22** significantly up-regulated P21 and P53 genes (Fig. 2). Upon establishing these promising effects, we set out this study to investigate the drug resistance triggered by these new heterosteroids through studying their effects on miR-34a, miR-98 and miR-214 expression (Fig. 2). Tamoxifen is considered as a selective estrogen

Fig. 1 Chemical structure of the newly synthesized heterosteroids

Table 1 The in vitro cytotoxic activity of the newly synthesized compounds on MCF-7 cancer cell line

Compd. no.	IC ₅₀ (μM)	Compd. no.	IC ₅₀ (μM)
4	9.6	18	26
6	4.8	21	29
7	23.3	22	32
13	23.6	24	18
Tamoxifen	4	Tamoxifen	4

IC₅₀, concentration required to inhibit cell viability by 50%

receptor modulator that targets estrogen receptor itself. Acquired resistance to tamoxifen is oftenly developed. However, the exact mechanism for the developed resistance is not fully understood. This is due to the incomplete understanding of signaling transduction pathways and components affecting cell proliferation, survival, and death in addition to their estrogen-mediated regulation in breast cancer and complexity of such signaling pathways that are closely related [24].

Recently, miRNAs were figured out as a key regulator of drug resistance to different chemotherapeutic agents. From these miRNAs, miR-34a family, which gained great interest because its participation with a functional role in tumor suppressor pathway. This was due to it is directly down-regulated by P53 [26–31]. In addition to, miR-34a targets many mRNA, from which MET, the proto-oncogenic hepatocyte growth factor. Besides, BCL-2, SIRT1, CDK and CCND1 were also reported as targets for miR-34a [14, 29, 32]. Surprisingly, high levels of miR-34a were associated with increased resistance in MCF-7 breast cancer cells to docetaxel drug [33]. In the current study, miR-34a expression level was insignificantly increased in tamoxifen treated MCF-7 cells as compared to control cells. Building on the previous work, this finding indicates increased resistance in MCF-7 against tamoxifen drug. However, cells treated with compounds 4, 6, 7, 13, 18, 21 and 24 showed a significant reduction in miR-34a expression levels (Fig. 3). Compound 4, 7, 18 and 24 reduced the expression levels of CCND1, Survivin, BCL-2 and CDC2 significantly. In previous study, both BCL-2 and CCND1

Fig. 2 Molecular regulation of new heterosteroids on apoptotic genes and miRNAs. A part of this figure was quoted from Elmallah and Micheau [25]

Fig. 3 The effect of new heterosteroids on miR-34a expression in MCF-7 cells, * $P < 0.05$

expression levels were reduced in MCF-7 docetaxel-resistant cells, parallel to miR-34a increase [33]. Hence the decrease in miR34a expression levels in this study could be attributed to the reduction in drug resistance to the newly synthesized compounds. Regarding compound 21 which was previously established to up-regulates P53 and P21 expression levels [23], it caused significant reduction in miR-34a expression levels. As mentioned previously, P53 has a down-regulating effect on miR-34a transcription [26–31, 34, 35]. Consequently, we could attribute the decrease in miR-34a expression levels in MCF-7 treated cells with compound 21 to its up-regulation by P53 levels. Like miR-34a, both miR-98 and miR-214 have a positive role in inducing apoptosis and reduction of proliferation [22, 36]. Similarly, like miR34a, tamoxifen treated cells

showed increased expression levels of both miR98 and miR-214, however, this increase was significant in this case (Figs. 3, 4, 5). On the contrary, most of our newly synthesized heterosteroids showed a significant reduction in expression levels of miR-98 and miR-214. miR-98 is one of let7 members. These members of let7 family target similar genes and have similar functions. They have a well established role as a tumor suppressor miRNAs [37]. The expression levels of both miR-98 and miR-214 were reported to be reduced in cancerous cells [38, 39]. MCF-7 treated with compounds 6, 7, 13, 18, 21 and 24 showed a significant decrease of miR98 expression levels. Nevertheless, this increase did not reach significance in cells treated with compound 13. On the contrary, cells treated with compound 22 showed a significant elevation in miR-98 expression levels as compared to control cells. This

Fig. 4 The effect of new heterosteroids on miR-98 expression in MCF-7 cells, * $P < 0.05$

Fig. 5 The effect of new heterosteroids on miR-214 expression in MCF-7 cells, * $P < 0.05$

elevation could be a compensatory mechanism by which the cells promote apoptosis, due to the positive role played by miR-98 in promoting apoptosis and reducing cell proliferation [36]. Likewise, MCF-7 cells treated with compounds 4, 7, 13, 18, 21 and 22 showed significant reduction in miR-214 expression levels (Figs. 4, 5). However cells treated with compound 6 showed a significant elevation in miR-214 expression levels as compared to control cells. miR-214 has a positive role in promoting apoptosis [22]. Building on this well established effect; we could consider the elevation in miR-214 expression levels as a cofactor in accelerating apoptosis. These results suggest that miR-98 and miR-214 levels are reduced, similar to miR-34a, in MCF-7 treated with the new heterosteroids. Building on this finding, we could postulate that both miR-98 and miR-214 are similar to miR-34a, which was found to be up-regulated in drug resistant cells and decreased upon the reduction of drug resistance. Our results are in line with the study of Kastl et al. [33] who stated that miR-34a expression was increased in MCF-7 docetaxel-resistant breast cancer cells and docetaxel response could be altered upon miR-34a modulation.

Conclusion

This study clarified that the newly synthesized heterosteroids have a lowered drug resistance response on MCF-7 as compared to tamoxifen. This effect was monitored through the reduced levels of miR-34a, miR-98 and miR-214 expression levels. These results provide evidence on the promising effects of these new heterosteroids as a potent anticancer drug against MCF-7 breast cancer cells.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

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