The ATP Assay, but not the MTT assay, Detects Further Cytotoxicity of the Combination of Anthracycline-based Therapy with Histone Deacetylase Inhibitor (Valproic Acid) in Breast Cancer Cells

[FAT testi, MTT Testinin Aksine, Meme Kanseri Hücrelerinde Antrasiklin-bazlı Tedavinin Histone Deasetilaz İnhibitörü (Valproik asit) ile Kombinasyonunun Yarattığı Daha İleri Düzeydeki Sitotoksisiteyi Tespit Edebilmektedir]

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ABSTRACT

Purpose: It has been investigated that whether or not the combination of valproic acid (a histone deacetylase inhibitor) with anthracycline-based chemotherapy (FEC: 5-fluorouracil+epirubicine + cyclophosphamide) would change the cytotoxic effects of FEC in breast cancer cells.

Methods: The effect of valproic acid and its combination with FEC has been tested on MDA-MB-231 and MCF-7 human breast cancer cell lines. Anti-growth effects of treatments were determined by the MTT and ATP assays, while the detection of apoptosis was performed by the caspase-cleaved cytokeratin 18 assay.

Results: Valproic acid treatment had anti-growth effect on the cell lines used at clinically achievable dose (0.6 mM). According to the MTT assay, the combination of valproic acid with different doses (50 – 200% Test Drug Concentration) of FEC did not result in any significant change over FEC-only treatment in both cell lines. However, according to the ATP assay, there has been found that the combination of 100% Test Drug Concentration FEC with valproic acid yielded more efficacy compared to FEC-alone. FEC induced the apoptosis in MCF-7 cells but the addition of valproic acid to FEC did not enhance apoptosis.

Conclusion: According to the ATP assay, the use of valproic acid at the clinically achievable dose (0.6 mM) with different doses of FEC further increased the cytotoxic effect of FEC. However, this effect was not observed in the MTT assay. A caution should therefore be taken on the evaluation of the cytotoxic effect of valproic acid in cell lines.

Key words: valproic acid, breast cancer, apoptosis, FEC protocol, M30 antigen.

ÖZET

Amaç: Bu çalışmada, valproik asidin (histon deasetilaz inhibitörü) antrasiklin-bazlı kemoterapi (FEC:5-Fluorourasıl+Epirubisin+Siklofosfamid) ile kombinasyonu halinde, FEC tedavisinin meme kanseri hücreleri üzerindeki sitotoksik etkisini değiştirip değiştirmediği araştırılabilir.

Metotlar: Valproik asidin tek başına ve FEC ile kombinasyonunun insan MDA-MB-231 ve MCF-7 meme kanseri hücre soylardaki etkilerini test edildi. Tedavilerin hücre büyümeyini baskılayıcı yeteneği MTT ve ATP canlılık testleri ile, apoptozis ise kaspazalık sitokeratin 18 ölçümlerle belirlendi.

Bulgular: Valproik asidin tedavisinin MDA-MB-231 ve MCF-7 hücrelerinde etkileri klinik olarak ulaşılabilen dozda (0,6 mM) büyüme baskısı sağladığı bulundu. MTT testine göre, valproik asidin FEC’in farklı dozlarıyla (%50 – 200 Test İlaç Dozu) kombinasyonunun, her iki hücre soyunda da FEC’in tek başına olan etkisine göre farklılığa neden olmadığı bulundu. Oysa ATP testine göre, %100 Test İlaç Dozu FEC ile valproik asit kombinasyonu, FEC’in tek başına etkisine göre daha etkili bulundu. FEC’in MCF-7 hücrelerinde apoptozisi indüklediği halde valproik asit ilavesinin apoptozisi daha fazla artırılmadığı gözlemdi.

Sonuç: ATP testine göre, valproik asidin klinik olarak ulaşılabilen dozunda (0,6 mM) farklı dozlardaki FEC ile kombinasyonu FEC’in sitotoksik etkisini artırırdı. Fakat bu artış MTT testine göre saptanamamaktadır. Bu nedenle, valproik asidin hücre soylardaki sitotoksik etkisi değerlendirilirken dikkat edilmelidir.

Anahtar Kelimeler: valproik asit, meme kanseri, apoptozis, FEC protokolü, M30 antijeni.
Introduction

Breast cancer is one of the major issues in health care in terms of morbidity, mortality and therapy costs (1). Surgical resection, radiotherapy, endocrine therapy (tamoxifen, aromatase inhibitors), targeted therapy (Herceptin®, Avastin®), and multichemotherapy regimens with different drug combinations (e.g. CMF: cyclophosphamide plus methotrexate plus 5-fluorouracil, FAC: 5-fluorouracil plus doxorubicin plus cyclophosphamide, FEC: 5-fluorouracil plus epirubicin plus cyclophosphamide, taxanes) are the main therapy concepts in breast cancer care (2, 3). Adjuvant treatment of high risk breast cancer patients with anthracycline containing regimens has been proven to be highly effective for treating patients with advanced breast cancer (4).

Valproic acid (VPA; a branched short chain fatty acid) is a histone deacetylase (HDAC) inhibitor that has been used for decades in the treatment of patients with epilepsy and other neuropsychiatric disorders (5). HDAC inhibitors are a class of molecules that modify chromatin structure and regulate gene transcription and expression (6). HDAC inhibitors have been shown to cause growth inhibition in several malignant cell lines, including breast cancer (7, 8). As the safety profile of VPA is well-established, this HDAC inhibitor is an attractive candidate for development as an anticancer agent (9, 10). VPA has shown potent antitumor effects in a variety of in vitro and in vivo systems, by modulating multiple pathways including cell cycle arrest, apoptosis, angiogenesis, metastasis, differentiation, and senescence. These effects seem to be cell type specific which may depend also on the level of differentiation and the underlying genetic alterations (11).

We investigated a novel combination of drugs by employing a polychemotherapy regimen (FEC) with a VPA for the aim to improve the chemotherapeutic efficacy of breast cancer treatment. In the present report, we employed the human estrogen receptor negative, highly invasive breast cancer cell line MDA-MB-231 and the estrogen receptor positive, non-invasive breast cancer cell line MCF-7 to determine changes in cell growth and apoptosis as a consequence of novel epigenetic combination therapies.

Materials and Methods

Chemicals and Anticancer Drugs

VPA was obtained from Sigma (St. Louis, MO). 5-Fluorouracil (5-FU; EBEWE Pharma, Austria), 4-HC (4-hydroperoxycyclophosphamide, the active metabolite of cyclophosphamide; NIOMECH, Germany), and epirubicine (EBEWE Pharma, Austria) were obtained from the Pharmacy of the Uludag University Hospital, representing standard drug regimens conventionally used for breast cancer treatment. Stock concentrations of each drug were prepared either in phosphate buffer saline (PBS) or in the dilution buffer provided by the drug company. Working dilutions of the drugs were prepared from stock solutions by diluting them in the appropriate culture medium. For each drug, four different concentrations were used and defined as test drug concentrations (TDC). TDC were determined by pharmacokinetic/clinical information and clinical evaluation data (12). 100% TDC was defined as a percent plasma drug concentration assayed after standard FEC dose administration in cancer patients (13). Hereby, 100% TDC values (in µg/mL) were defined as follows: 5-FU: 22.50, epirubicine: 0.50, 4-HC: 3.0. Drug concentrations used for in vitro experiments were 200, 100 and 50% of TDC.

Cell Culture and Drug Treatment

Breast cancer cell lines MCF-7 (estrogen receptor-positive) and MDA-MB-231 (estrogen receptor-negative) were cultured in RPMI 1640 supplemented with penicillin G (100 U/ml), streptomycin (100 µg/ml), L-glutamine, and 10% fetal calf serum (Invitrogen, Paisley, UK) at 37°C in a humidified atmosphere containing 5% CO₂. For the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay, MCF-7 and MDA-MB-231 cells were seeded per well of a 96-well plate in 200 µl culture medium in triplicates at a density of 5x10³ cells and at a density of 10⁴ cells for the ATP assay. After overnight incubation, media were replaced by fresh ones with or without the test drugs. For minimum growth control, cells were incubated with 1 mM H₂O₂ (positive control for cell death). Untreated cells grown in culture medium were used for the maximum growth control (negative control for cell death). Cells were treated for 48 h with 0.6, 0.3 and 0.15 mM VPA or with VPA plus increasing doses of FEC (50, 100, and 200% TDC).

MTT Assay

The MTT cell growth assay was previously described (14). In brief, MTT was supplied as a stock solution (5 mg/ml PBS, pH 7.2) and sterile-filtered. At the end of the treatment period with 3 different TDC in triplicates (48h) plus untreated control sets, 25 µl of MTT solution was added to each well and then, after another 4 h incubation at 37°C, 100 µl of solubilizing buffer (10% SDS dissolved in 0.01 N HCl) was added to each well. After overnight incubation, the absorbance was determined by an ELISA plate reader (FLASH Scan S12, Analytik Jena, Germany) at 570 nm as a read-out for cell growth. The viable cells produced dark blue formazan products, whereas no such staining was observed in dead cells. Cell growth of treated samples (SampleV) was calculated in reference to the untreated control cell line, which was defined as 100% growth (maximal growth, MaxG). The H₂O₂-treated cell control was defined as minimal growth reference (minimal growth, MinG). Thus, the degree of inhibition of drug-treated cells is expressed as the percentage of the untreated cell control using the formula: growth (%) = [100 x (SampleV - MinG)/(MaxG - MinG)].
**ATP Assay**

The ATP assay uses the highly sensitive “firefly” reaction to determine the level of cellular ATP as a surrogate marker for the number of viable (ATP-producing) cells (13). At the end of the treatment period with three different TDCs in triplicate (48 h), the ATP content was determined according to the manufacturer’s recommendation (DCS Innovative Diagnostika-Systeme, Hamburg, Germany). For this, ATP was extracted from the cells, placed into white microtiter plates, and then luciferin-luciferase added. Luminescence was determined by a count integration time of 1 s in a luminometer (Bio-Tek, Winooski, USA). The results are given as relative light units (RLU).

**Caspase-cleaved Cytokeratin 18 (M30-Antigen) Detection**

To detect the M30 cytokeratin fragment, a solid phase, two-site immunosorbent assay was used (M30-Apoptosense ELISA kit, Peviva, Bromma, Sweden). Briefly, test samples were placed into wells of a microtiter plate coated with a mouse monoclonal antibody to cytokeratin 18 as the catcher antibody. Samples were incubated for 4 h at room temperature on a shaker at 600 rpm, followed by addition of an M30-directed horseradish peroxidase-conjugated mouse monoclonal detection antibody, TMB substrate (20 min), peroxidase stop solution, and absorbance was determined at 450 nm (FLASH Scan S12, Analytik Jena, Germany).

**Statistical Analyses**

All statistical analyses were performed using the SPSS 13.0 statistical software for Windows. The TDC were plotted against the corresponding cell growth values using one-way analysis of variance (ANOVA). Mann–Whitney’s U-test was used to analyze the association between the methylation statuses of the assessed genes. A value of \( p < 0.05 \) was considered statistically significant. Results are expressed as mean values plus/minus standard deviation.

**Results**

1. **Anti-growth effect of VPA on MCF-7 and MDA-MB 231 cells.**

   We first used MTT assay to measure the growth inhibition of MDA-MB-231 and MCF-7 breast cancer cell lines in various concentrations of VPA (0.15, 0.3, 0.6 mM) that have previously been shown as plasma concentrations. As shown in figure 1A, according to the MTT assay, VPA showed no significant inhibitory effect on the growth of MDA-MB-231 cells after 48 h. In MCF-7 cells, VPA significantly inhibited the growth at the highest dose (0.6 mM) \( (p<0.05) \). Alteration in proliferation can also be assessed by measuring the intracellular ATP levels in cells. VPA did not cause any change in the intracellular ATP levels of MCF-7 cells, whereas it reduced the ATP levels in MDA-MB-231 cells in a dose-dependent manner (Figure 1B). At 0.6 mM concentration of VPA, significant effects on cell growth of MDA-MB 231 cells \( (p<0.05) \) were observed. Generally, the intracellular ATP level was higher in MDA-MB-231 cells than that in MCF-7 cells.

2. **Effect of anthracycline-containing therapy regimen and VPA on MDA-MB-231 breast cancer cell growth.**

   Figure 2 shows the growth levels of MDA-MB-231 cells after 48 h treatment with 0.6 mM VPA in combination with FEC at three different doses (200%, 100% and 50% TDC). Treatment with FEC significantly increased growth inhibition in a dose dependent manner. When VPA was combined with FEC, the cell growth was not significantly changed in compared to FEC alone (Figure 2A). At the same time, FEC decreased the ATP levels in a dose-dependent manner. When VPA was combined with FEC, the intracellular ATP levels were further decreased, compared to FEC alone (Figure 2B). Especially,
at 100% TDC, this effect was statistically significant (p<0.05).

3. Effect of anthracycline-containing therapy regimen and VPA on MCF-7 breast cancer cell growth.

The growth levels of MCF-7 cells after 48 h treatment with 0.6 mM VPA in combination with different doses of FEC (200%, 100% and 50% TDC) were investigated by MTT assay. The cell growth greatly decreased after FEC treatment at all doses used. Therefore, the addition of VPA with FEC did not provide any further effect in growth (Figure 3A). In similar, ATP levels also greatly decreased after all doses of FEC so that we did not observe any further growth inhibition effect upon VPA addition (Figure 3B).


We examined the effects of different combinations of VPA, FEC and combination treatments (for 48 h) on apoptosis by measuring the caspase cleaved cytokeratin-18 (M30 antigen) which is a well-known marker for apoptosis. As shown in Figure 4A, we found that M30 antigen levels were not significantly changed after the treatments used in MDA-MB-231 cells. On the other hand, in MCF-7 cells, M30 antigen levels were increased after 100% TDC FEC and 100%TDC FEC + 0.6 mM VPA treatments compared to control (Figure 4B).
However, the detection of intracellular ATP levels did not confirm this inhibition of growth. This may be because of the much lower levels of ATP in MCF-7 cells, compared to those in MDA-MB-231 cells. Therefore, we think that the ATP assay may not be suitable for the assessment of growth in MCF-7 cells.

In contrast, the intracellular ATP levels significantly decreased after VPA treatment at clinically achievable dose (0.6 mM) in MDA-MB 231 cells at 48 h. Why we did not observe significant cytotoxic effect of VPA could be that the dose used in the present study is much lower than those reported in the literature. For example, it was previously reported that VPA caused cytostatic and cytotoxic effects at only higher doses in MCF-7 (2.34 mM) and MDA-MB 231 (4.14 mM) in breast cancer cells (22). Cytotoxic effect was, in fact, reached at VPA doses higher that 3.1 mM that is reported to be related to the development of severe side effects (23).

In the present study, treatment of both cell lines with various doses of FEC increased the inhibition of growth, assayed by the MTT and ATP-assays. When VPA combined with FEC, the intracellular ATP levels did not further decrease compared to FEC alone in MCF-7 cells. But, this is because the doses of FEC used were too toxic. Therefore, we did not observe any further cytotoxic effect in this cell line after the addition of VPA. Furthermore, the level of ATP was also relatively much lower in this cell line, resulting in inappropriate use of ATP assay as a cytotoxicity assay. In fact, all the cytotoxicity assays may not perfectly fit into all type of cell lines because of the differences in their metabolic activities. Furthermore, the effects of drugs may result in different efficacy according to the assay used. For example, Ulukaya et al. reported that the MTT assay yielded a relatively lower result of growth inhibition than the ATP assay depending on the chemotherapeutic drugs tested (14). Taking this fact into account, the MTT assay did not show any further increase in the inhibition of growth after the addition of VAP to FEC, while the ATP assay did. The inconsistency between the cytotoxicity results in the present study proves that all the cytotoxicity tests may not yield the same cytotoxicity levels although the same agents are used under the identical conditions.

In our study, according to the MTT assay, VPA treatment decreased the growth of MCF-7 cells by about 10-15%. However, the detection of intracellular ATP levels did not confirm this inhibition of growth. This may be because of the much lower levels of ATP in MCF-7 cells, compared to those in MDA-MB-231 cells. Therefore, we think that the ATP assay may not be suitable for the assessment of growth in MCF-7 cells.

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The combination of VPA with various doses of FEC further increased the inhibition of growth in MDA-MB 231 cells but only 100% TDC FEC reached the statistically significant level. This may be due to the fact that because 200% TDC FEC was too toxic to the cells, we did not observe any further cytotoxic effect of additional VPA at this concentration of FEC. Regarding to the 50% TDC of additional FEC, there has still been some further effect but it was not significant. This may be due to relatively weaker cytotoxic effect of FEC on the cells, compared to the 100% TDC that shows a noticeable difference. In agreement with our results, VPA potentiates the antiproliferative actions of tamoxifen, raloxifen, fulvestran and letrozol in breast cancer cell lines. In addition, Arakawa
et al. showed synergistic interaction between camptothecin and VPA with respect to the induction of antiproliferative effect and apoptosis in MCF-7 cells (24). Yap et al. informed that the VPA interact with anticancer drugs and it may lead to risk regarding tumors (25). When the VPA used with cisplatin chemotherapy, it has been found that VPA altered protein binding or increased volume of distribution of the drug and toxicity (26-28).

To determine cell death by apoptosis, a monoclonal antibody (M30) specific to caspase-cleaved forms of cytokereatin-18 released from apoptotic cells is available. These fragments are therefore conveniently determined by ELISA. Such an assay was successfully used to evaluate the response to cytotoxic anticancer drugs in cancer patients (29, 30). In our study, VPA, FEC and VPA+FEC treatments increased the apoptosis in MCF-7 cells. Interestingly, combination therapy did not further increase the apoptosis-inducing effect of FEC. But this does not mean that the cytotoxicity did not further increase. In this case, the cytotoxicity may result from necrosis, rather than apoptosis. In fact, MDA-MB 231 cells were died via necrosis after FEC treatment, which was observed by microscopic evaluation.

Taken together, according to the ATP assay, VPA treatment at the clinically achievable dose may increase the effects of FEC therapy in breast cancer cells although the MTT assay did not confirm this

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References


