

Metronidazole affects breast cancer cell lines

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ABSTRACT

Purpose: The aim of our study was to evaluate the impact of metronidazole (MTZ) on cytotoxicity and DNA synthesis in MCF-7 (estrogen receptor positive) and MDA-MB-231 (estrogen receptor negative) breast cancer cell lines.

Material/Methods: Toxicity of MTZ was determined by MTT test. MCF-7 and MDA-MB-231 cells were incubated with metronidazole used in different concentrations for 24, 48 and 72 hours. The effect of MTZ on DNA synthesis was measured as [3H]-thymidine incorporation.

Results: We showed that MTZ in concentration 250 µg/ml significantly increases the growth of MCF-7 cell lines after 24 hours of incubation, but it reduces cell viability in concentrations 1 and 10 µg/ml 72 hours after the drug application. Significant increase of MDA-MB-231 cell viability was obtained in MTZ concentration of 250 µg/ml after 24 and 72 hours. The increase of [3H]-thymidine incorporation in MCF-7 cell line treated with MTZ in concentration 250 µg/ml was statistically significant after 24 hours. Great suppression of cell proliferation was obtained in MDA-MB-231 breast cell line after application of the following concentrations of MTZ: 0.1 µg/ml (after 24 hours) and 0.1, 10, 50, 250 µg/ml (after 72h).

Conclusions: We found that metronidazole exerts different dose- and time- dependent effects on human breast cancer cell lines characterized by presence or absence of estrogen receptors. We suggest that these discrepancies may be influenced by the estrogen signaling.

Key words: metronidazole, MCF-7, MDA-MB-231, cytotoxicity, DNA synthesis

INTRODUCTION

Breast cancer represents a major health problem in European and North American women with over 1 million new cases reported every year and it is the second cause of death from cancer [1]. Currently many different chemotherapeutics are approved in breast cancer treatment therapy. Their anticancer potential may be related to the presence or absence of estrogen receptors. Some drugs used to treat various diseases were found to be carcinogenic and therefore the knowledge

about types of cancers development can be linked to earlier metronidazole (MTZ) treatment is of great importance. It is especially essential that in many cases women with breast cancer were treated with MTZ before diagnosis due to some bacterial infections (gardiiasis, trichomoniasis or *Helicobacter pylori* infection) [2,3].

Metronidazole, a synthetic derivative of nitroimidazole is active against most obligate anaerobes [2,4,5] and so is used in the treatment of the above infections and also in surgery, especially breast cancer and colon cancer surgery. Preoperative prophylaxis is divided into: the method of single-

dose, given just before surgery, and short-time method, that is one dose before surgery, and two more within 24 hours [6,7]. It has been demonstrated that preoperative prophylaxis reduces the risk of septic complications, which could lead to failure of the operation, increased mortality and treatment costs [6,8,9]. MTZ is generally well tolerated, although there are concerns that the drug may have mutagenic and carcinogenic effects [10]. MTZ binds to DNA causing its damage, on the other hand it is capable of inducing gene mutations in mammalian cells [11]. In the majority of experiments, the doses were much higher than those used in the human infections treatment [4]. Menendez *et al.* [11] determined the degree of DNA damage in lymphocytes of healthy people treated with therapeutic doses of the drug and they found an inverse correlation between the degree of DNA damage and the concentration of MTZ in plasma [11]. MTZ may be carcinogenic in mice and rats if used in large doses for an extended period of time [3]. Although carcinogenicity data in humans are inconclusive. Metronidazole effects, besides those commonly known, were also studied in cell cultures. Mohindra *et al.* [12] studied MTZ effects on cell viability in vitro and in the presence of air or nitrogen. The results of these experiments indicated an increased toxicity of the drug under hypoxic conditions. All of the cells: CHO (Chinese hamster ovary), HeLa (derived from cervical cancer) and human marrow cells responded similarly to increasing concentrations of MTZ in medium [12]. Based on this information and the clinical situation when women with breast cancer are exposed to metronidazole, the aim of our study was to evaluate the effect of MTZ on MCF-7 (estrogen receptor (ER)-positive) and MDA-MB-231 (estrogen receptor (ER)-negative) cell cultures of breast cancers. Metronidazole was used in different concentrations and time of exposition. Evaluation of cytotoxicity and DNA synthesis will allow us to draw the conclusions about the impact of metronidazole on growth and proliferation of breast cancer cells characterized by presence or absence of estrogen receptors.

MATERIAL AND METHODS

Drug

Metronidazole purum (>98%) was supplied by Sigma Chemical Co (St. Louis, Mo). The drug was dissolved in DMSO and it was applied to the cell culture in concentrations: 0.1, 1, 10, 50, 250 µg/ml (0.58 µM, 5.8 µM, 58 µM, 292 µM, 1.46mM, respectively). After 24 and 48 hours cell culture was rinsed three times with PBS and MTZ was applied again to observe its effect after 48 and 72 hours.

Tissue culture

All studies were performed on breast cancer MCF-7 and MDA-MB-231 cell lines, purchased in American Type Culture Collection (Manassas, Virginia, USA). The cells were maintained in DMEM with GlutaMax I supplemented

with 10% fetal bovine serum (FBS), 50 U/ml penicillin, 50 mg/ml streptomycin at 37°C in a 5 % CO₂ incubator. Cells were counted in a hemocytometer and cultured at 1 x 10⁵ cells per well in 2 ml of growth medium in 6 well plates (Sarstedt, USA). Cells reached confluence at day 4 and such cells were used for the assays. Cells were used in the 8th to 14th passages.

Cytotoxicity assay

Toxicity of MTZ was determined by method of Plumb *et al.* [13]. Cells were maintained as described above. After 24, 48 or 72 hours of incubation with MTZ the culturing medium was discarded, the cells were rinsed three times with PBS. Then the cells were incubated for 4 hours in 2 ml of PBS with 50 µl of MTT (5mg/ml). Medium was removed from the wells, and the cells were dissolved in 200 µl of DMSO with 20 µl of Sorensen's buffer (0.1 mol/l glycine with 0.1 mol/l NaCl equilibrated to pH 10.5). The absorbance was recorded with spectrophotometer (Fisher Scientific) at wavelength of 570 nm. Values are described as a percent of control.

[3H]-thymidine incorporation.

To examine the effect of MTZ on cell proliferation, cells were seeded in 24 well plates at 1 x 10⁵ cells/well with 1 ml of growth medium. After 48 hours to subconfluent cells various concentrations of the drug and 0.5 mCi of [3H]-thymidine were added. The incubation was continued for 24, 48 or 72 hours at 37°C. Cells were rinsed 3 times with PBS, solubilized with 1 ml of 0.1 mol/l sodium hydroxide containing 1% SDS, then scintillation fluid „Ultima Gold XR” was added and incorporation of the tracer into DNA was measured in scintillation counter.

Statistical analysis

The results were analyzed by Statistica 9.0 using ANOVA tests at a significance level of $p < 0.05$.

RESULTS

Viability of MCF-7 and MDA-MB-231 cell lines treated for 24, 48 or 72 hours with different concentrations of metronidazole (0.1, 1, 10, 50 and 250 µg/ml) is shown on figures as a percent of control. After 24 and 48 hours the viability of MCF-7 cells increased compared to cells without the drug and it grew to 122.17% when MTZ was used in the concentration 250 µg/ml ($p < 0.05$) noted only after 24 hours of observation period. Reduced viability of cells after 72 hours was significant for 1 and 10 µg/ml of MTZ. We observed significant ($p < 0.05$) decrease in viability of MCF-7 cells in time (comparing to the first time of observation) treated with MTZ at concentrations: 0.1, 1 and 10 µg/ml. The greatest survival of MDA-MB-231 cells reached 124.2% after 24 hours (250 µg/ml) and 109.45% (250 µg/ml) after 72 hours of exposition to MTZ ($p < 0.05$).

The significant increase of [3H]-thymidine incorporation in MCF-7 cell line was after 24 hours and equaled: 104.9% ($p<0.05$) at MTZ concentration 250 $\mu\text{g/ml}$, comparing to control cells, and also after 48 hours - 117.33%, but there was no statistical significance (Fig. 1). Suppression of cell proliferation was obtained in MDA-MB-231 breast cell line at the following concentrations of MTZ: 0.1 $\mu\text{g/ml}$ (after 24 hours; $p<0.05$), 0.1 (after 72h; $p<0.01$), 10, 50, 250 $\mu\text{g/ml}$ (after 72h; $p<0.001$). We found statistically significant increase of DNA synthesis in these cells exposed to 250 $\mu\text{g/ml}$ MTZ after 24 hours. Metronidazole at the concentration 250 $\mu\text{g/ml}$ was found to inhibit thymidine incorporation into DNA in MDA-MB-231 cells in time dependent manner (Fig. 2).

DISCUSSION

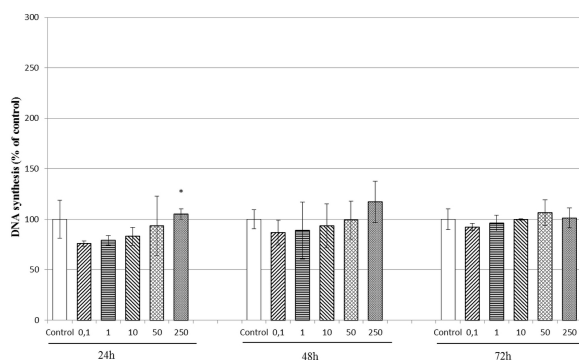
The rate of mortality from the breast cancer is still very high [14]. Looking for a proper treatment, people almost forget of satisfactory prophylaxis. Moreover, the knowledge about the drugs with carcinogenic potential is still poor. When carcinogenicity is found in animal models, it is important to determine whether it is also true for humans, as not all chemicals known to cause tumors in animals also induce tumors in humans [15].

Literature data may suggest that MTZ is carcinogenic [2-4,16]. The drug is used in breast cancer surgeries while removing breast tumors, and also it may be administrated in some infections: gardiasis, trichomoniasis or *Helicobacter pylori* infection. Thus the question, if MTZ can contribute to the spread of cancer cells or increase cell proliferation is still open.

Data presented here show that survival of MCF-7 (ER-positive) cells treated with metronidazole is dependent on the concentration of the drug and the time of observation. We found that MCF-7 cells respond to MTZ with significant

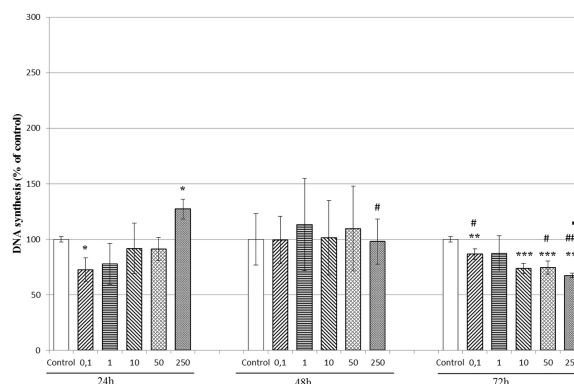
increase in survival after first day (250 $\mu\text{g/ml}$) of exposition to the drug. Unexpectedly, MCF-7 cells survival was significantly decreased after 72 hours of exposition to MTZ used only at concentration 1 and 10 $\mu\text{g/ml}$. An increased cell viability after 24 hours, but also after 48 hours and the extinction of this effect after 72 hours may be due to loss of MTZ activity or drug degradation, or its toxic effect. We also found that increased concentration of MTZ enhanced survival of MDA-MB-231 (ER-negative) cells independently of the time of exposition to the drug. Generally, we suggest that MTZ may enhance survival of breast cancer cells estrogen-independent (MDA-MB-231) or estrogen-dependent (MCF-7) when it is used in high concentration. The viability results were similar in both cell lines after 24 and 48 hours, which allows us to deduce, that the presence of estrogen receptors is irrelevant. However, we found some differences after 72 hours of experiment between MCF-7 and MDA-MB-231 cells viability. Significant reduction of MCF-7 cells viability after small doses of MTZ (1, 10 $\mu\text{g/ml}$) and no changes in MDA-MB-231 cells viability could indicate that the presence of estrogen receptors (ER) may sensitize the cells to MTZ. MTZ concentrations at 1 and 10 $\mu\text{g/ml}$ reflect better the clinical reality, because such concentration range was detected in colon cancer cells in humans exposed to MTZ used as a prophylactic before surgery (under publication). Despite the fact that our results show differences in cell lines sensitivity to the drug, we cannot point the reason for such differences. Our results are unique, even though they are performed on the cell cultures, because only few studies tried to find a correlation between MTZ and cancer. Although, there have been some studies on cancer induction. Falagas *et al.* [3] studied the occurrence of cancer after taking metronidazole and compared the results with a group of people who were not receiving this drug. The main conclusion of their work was that the incidence of cancer among patients taking MTZ was almost identical to that in a control group of equivalent

Figure 1. Effect of metronidazole on [3H]-thymidine incorporation into MCF-7 cell line (n=6-9).



* $p<0.05$ vs. control group without metronidazole

Figure 2. Effect of metronidazole on [3H]-thymidine incorporation into MDA-MB-231 cell line (n=6-9).



* $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs. control group without metronidazole
 # $p<0.05$, ### $p<0.001$ vs. equivalent concentration of 24h group
 • $p<0.05$ vs. equivalent concentration of 48h group

Figure 3. Effect of MTZ on MCF-7 cell viability presented as a percent of control (n=6-9).

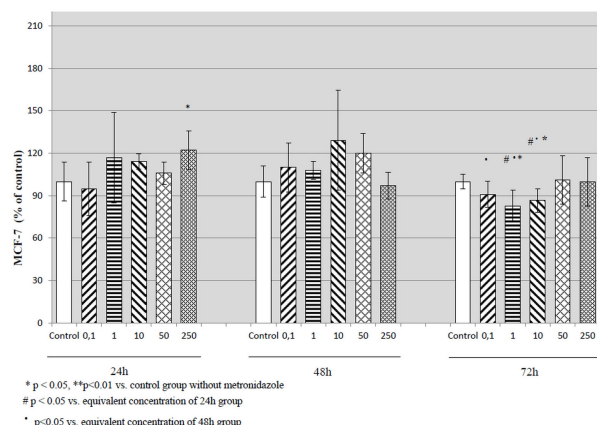
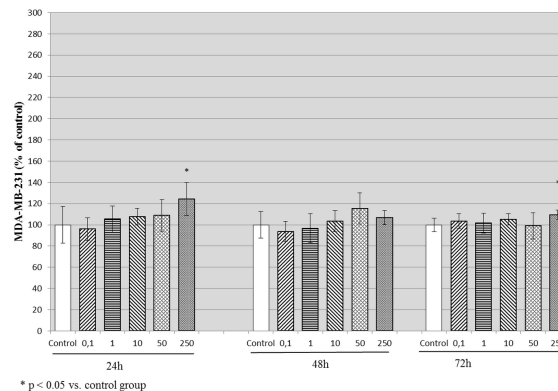


Figure 4. Effect of MTZ on MDA-MB-231 cell viability presented as a percent of control (n=6-9).



age, gender and years of study. Similarly, Friedman *et al.* [17] performed a study that lasted more than 9 years and included more than 2 million women, aged 20 and above, and had prescribed at least one antibiotic. 18,521 women developed breast cancer. The authors observed a slightly increased risk of cancer associated with antibiotic [17].

Friedman *et al.* [15] assessed the risk of breast cancer among 8 drugs that induce mammary tumors in experimental animals. In their cohort, MTZ showed statistically significant yet very small increases in relative risk (1.07 to 1.13) [15]. In the next study, performed on animal models the authors attempted to link the old and new cancer cases with the drug. The odds ratio was not elevated for cervical cancer, which has been suspected to be associated with MTZ as it is often used in trichomonal vaginitis treatment. There was weak confirmation of suspected association of MTZ with breast cancer. The odds ratio (95% confidence interval) for breast cancer was 1.13 among 498 exposed cases [18].

Although, based on clinical data MTZ does not induce cancer, we still do not know about its effect on oncological patients. MTZ is administered prophylactically to patients before and during surgery. Therefore, the 24 hour exposition to the drug seems to be important, as confirmed by literature data. Stewart *et al.* [19] combined metronidazole with mitomycin-C in the treatment of breast cancer and they suggested that metronidazole may potentiate the effect of chemotherapy. Patients were taking MTZ (1.5 g/m²) orally 12 and 1 hour before chemotherapy and again 6 and 24 hours after mitomycin-C. None of estrogen receptor positive patients responded to the drug, while negative estrogen receptor individuals had the highest response rates. Other doses of metronidazole might be worthy to test as it has the ability to potentiate the effect of chemotherapy [19]. Additionally, our results show that MTZ (250 µg/ml) increased survival of breast cancer cells after 24, 48 hours of administration and this may affect cancer recurrence even if this drug (1 and

10 µg/ml) caused the decrease of (after 72 hours) estrogen dependent breast cancer cells (MCF-7) viability. In spite of this we have shown that estrogen dependent breast cancer cells respond to MTZ in different manner than estrogen independent ones but only after a long-lasting drug exposition. Experiments performed on animals showed that MTZ causes breast, lung, spleen and liver tumors, ovarian cysts in female rats, hemangioma of the liver, lung and liver tumors, Leydig cell tumors or pituitary adenoma in male rats. The increase in tumor incidence was statistically significant only in case of females, which may have been affected by female sex hormones or differences in the absorption of metronidazole by females and males [2,4]. Nevertheless, we cannot forget that a cell line is not the same as a living organism, so it should not be blindly compared.

Because MTZ damages DNA and the effects of estrogens and anti-estrogens on DNA synthesis in hormonally responsive breast cancer cell lines have already been documented [20,21] we decided to study the influence of MTZ on DNA synthesis in MCF-7 and MDA-MB-231 cells.

The results of DNA synthesis after 24 hours of exposition to MTZ indicate a reduction (MTZ = 0.1 µg/ml) or enhancing (MTZ = 250 µg/ml) in MDA-MB-231 cell proliferation. This fluctuation of DNA synthesis is consistent with viability of cells in the same time, e.g. the enhance of thymidine incorporation in ER- cells after 24 hours is consistent with the enhancement of the viability (MTZ = 250 µg/ml).

There was no significant increase in MCF-7 and MDA-MB-231 cell proliferation after 48 hours of exposition. However after 72 hours a significant reduction in DNA synthesis was observed only in MDA-MB-231 cells treated with MTZ. Significant inhibition of DNA synthesis in MDA-MB-231 cell line after 3 days of exposure to MTZ and simultaneous lack of changes in the cell survival may indicate an increased number of cells in a particular cell cycle phase and thus may suggest tumor cell cycle arrest in S phase.

Moreover, prolonged observation with the apparent trend of decreasing thymidine incorporation would eventually result in reduced number of living cells.

Because this effect was observed only in ER - independent cancer cells, this may indicate various reactions of cancer cells to MTZ. At the present stage of knowledge and based on the obtained results, we cannot explain the mechanism of this phenomenon.

Data on influence of MTZ on DNA synthesis in cancer cells is poor. Ito *et al.* [22] performed a study where MTZ is not significantly involved in the cell nuclei activity based on the thymidine incorporation assay. Authors claim that in tumors, neoplastic cells can outgrow under conditions of oxygen deficiency, so that hypoxic tumor cells are metabolically active. In their animal model, tumor oxygen tension was determined to be 3.2-6.0 mmHg, while normal muscle tissue had 30-40 mmHg. Hypoxia induces angiogenesis and thus increases the invasive potential and the possibility of occurrence of metastases.

Metronidazole and other nitroimidazoles are enzymatically reduced and thus activated within viable hypoxic cells [22]. Therefore, MTZ penetrates into the hypoxic environment of the tumor where may exert pro- and anti-angiogenic effects, by changing cancer proliferation, and influence by that the process of metastasis. Our next study will concentrate on this problem.

Based on the new trend in pharmacotherapy, physicians should try to avoid prescribing MTZ for women with some types of breast cancers, presenting certain estrogen receptor status. This strategy would allow to minimize the risk of carcinogenic effect of MTZ.

CONCLUSIONS

We found that MTZ exerts different dose- and time- dependent effects on human breast cancer cell lines characterized by presence or absence of estrogen receptors. We suggest that these discrepancies may be influenced with the estrogen signaling.

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