Metronidazole Decreases Viability of DLD-1 Colorectal Cancer Cell Line

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Abstract

The aim of our study was to evaluate the impact of metronidazole (MTZ) on DLD-1 colorectal cancer cell (CRC) line. Toxicity of MTZ was determined by MTT test. Cells were incubated with MTZ used in different concentrations for 24, 48, and 72 hours. The effect of MTZ on DNA synthesis was measured as [3H]-thymidine incorporation. The morphological changes in human DLD-1 cell line were defined by transmission electron microscope OPTON 900. The influence of MTZ on the apoptosis of DLD-1 cell lines was detected by flow cytometry and fluorescence microscopy, while cell concentration, volume, and diameter were displayed by Scepter Cell Counter from Millipore. Our results show that cell viability was diminished in all experimental groups in comparison with the control, and the differences were statistically significant. We did not find any significant differences in [3H]-thymidine incorporation in all experimental groups and times of observation. Cytofluorimetric assays demonstrated a statistically significant increase of apoptotic rate in MTZ concentrations 10 and 50 $\mu\text{g}/\text{mL}$ after 24 hours; 0.1, 10, 50, and 250 $\mu\text{g}/\text{mL}$ after 48 hours; and in all concentrations after 72 hours compared with control groups. In the ultrastructural studies, necrotic or apoptotic cells were occasionally seen. In conclusion, MTZ affects human CRC cell line viability. The reduction of cell viability was consistent with the apoptotic test.

Key words: apoptosis, cell viability, DNA synthesis, DLD-1, electron microscopy, metronidazole

Introduction

Colorectal cancer (CRC), which originates in epithelial structures of the large bowel mucosa, is one of the most frequent human cancer and a significant cause of morbidity and mortality in Western populations.^{1–3} Incidence of CRC has grown rapidly since 1990, and is influenced by eating and lifestyle habits that include increased meat intake and reduced fiber intake, alcohol consumption, or smoking.^{4,5} The treatment of CRC has undergone substantial improvements in the last 10 to 15 years in terms of screening, surgical management of resectable disease, and adjuvant chemotherapy. Moreover, 5-year survival for all patients has improved from only 50% to 62% during the last 25 years.⁶ Surgical removal of malignant disease is the main method of treatment for patients with CRC. However, the tumor resection is associated with high risk of perioperative infections, which are an im-

portant cause of morbidity and prolonged hospital stay. Numerous studies have shown that preoperative antibiotic prophylaxis reduces cases of septic complications after surgery of gastrointestinal tract.^{7,8} In surgical patients, antimicrobial prophylaxis is indicated particularly in the surgery of large intestine and rectum, where anaerobic bacteria, such as *Bacteroides fragilis*, predominate.⁹ Currently, there are many combinations of antibiotics and chemotherapeutic agents used in surgical prophylaxis.¹⁰ Therefore, the most commonly used regimens are cephalosporins and/or aminoglycosides, and metronidazole (MTZ). MTZ is an important drug for various infections that are induced by anaerobic bacteria.⁹ It is the principal treatment for several types of illnesses caused by protozoa and anaerobic bacteria such as *Helicobacter pylori* infections, amebiasis, giardiasis, and trichomoniasis.¹¹ MTZ is generally well tolerated; however there are concerns that the drug may have mutagenic and carcinogenic effects. MTZ is a

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potent mutagen in bacteria,¹² although its genotoxic potential in humans is still contradictory.¹³ With regard to carcinogenic potential of MTZ, the drug increases the incidence of lymphomas and adenomas in mice and colon cancer in rats.¹⁴ According to the International Agency for Research on Cancer (IARC), the evidence is sufficient to consider MTZ as an animal carcinogen, but insufficient for humans.¹⁵ Data available from epidemiological studies are inadequate to evaluate the relationship between exposure to MTZ and human cancer.¹⁵

Our previous study on patients with CRC showed increased accumulation of MTZ in the tumor tissue.¹⁶ Thus, MTZ is used in colon cancer surgery; the main objective of our investigation was to verify the possible effect of MTZ on DLD-1 colon cancer cell line when the dose is repeated.

Materials and Methods

Drug

Metronidazole purum (>98%) was supplied by Sigma Chemical Co.

The drug was dissolved in dimethyl sulfoxide (DMSO), and it was applied to the cell culture in the following concentrations: 0.1, 1, 10, 50, and 250 $\mu\text{g/mL}$ (0.58 μM , 5.8 μM , 58 μM , 292 μM , and 1.46 mM, respectively). After 24 and 48 hours, cell culture was rinsed thrice with phosphate-buffered saline (PBS), and MTZ was applied again to observe its effect after 48 and 72 hours. Cultured DLD-1 cells without the drug were used as a control.

Tissue culture

All studies were performed on colon cancer DLD-1 cell line, purchased in American Type Culture Collection. The cells were maintained in DMEM with GlutaMax I supplemented with 10% fetal bovine serum, 50 U/mL penicillin, and 50 mg/mL streptomycin at 37°C in a 5% CO₂ incubator. Cells were counted in a hemocytometer and cultured at 1×10^5 cells per well in 2 mL of growth medium in six-well plates (Sarstedt). 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 the method of Plumb et al.¹⁷ Cells were maintained as described earlier. After 24, 48, or 72 hours of incubation with MTZ, the culturing medium was discarded, and the cells were rinsed thrice with PBS. Then, the cells were incubated for 4 hours in 2 mL of PBS with 50 mL of MTT (5 mg/mL). Medium was removed from the wells, and the cells were dissolved in 200 mL of DMSO with 20 mL of Sorensen's buffer (0.1 M glycine with 0.1 M NaCl equilibrated to pH 10.5). The absorbance was recorded with a spectrophotometer (Fisher Scientific) at a 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 1×10^5 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 thrice with PBS, solubilized with 1 mL of 0.1 M sodium hydroxide containing 1% sodium dodecyl sulfate, then scintillation fluid "Ultima Gold XR" was added, and incorporation of the tracer into DNA was measured in a scintillation counter.

Flow cytometry

The detection of loss of membrane permeability and exposure of phosphatidylserine (PS) was detected using an Annexin V: FITC Apoptosis Detection Kit I (BD Pharmingen™). Cells incubated in the presence or absence of MTZ were harvested at 24, 48, and 72 hours. Cells were trypsinized and washed twice with cold PBS. Then, the cells were resuspended in 1×Binding Buffer at a concentration of 1×10^6 cells/mL. One hundred microliters of the solution (1×10^5 cells) was transferred to a 5 mL culture tube; then, 5 μL of FITC Annexin V along with 5 μL propidium iodide (PI) was added sequentially. The cells were vortexed and incubated for 15 minutes at room temperature (25°C) in the dark. Four hundred microliters of 1×Binding Buffer was added to each tube. The samples were analyzed by flow cytometry within 1 hour.

Fluorescence microscopy assay

Apoptotic and necrotic cells were detected using staining mixture (100 $\mu\text{g/mL}$ in PBS of acridine orange and 100 $\mu\text{g/mL}$ in PBS of ethidium bromide). Cells were examined for morphological features of apoptosis and necrosis (chromatin condensation, fragmentation, and apoptotic body formation) under a fluorescence microscope (Olympus CKC41, U-RLFT50) and photographed (400×total magnification).

Cell parameters

Cells were trypsinized and washed with PBS. The volume of 100 μL DLD-1 cells was suspended with diluent in order to receive a dilution of 1:20. Cell concentration, cell volume, and cell diameter were displayed by Scepter Cell Counter from Millipore.

Morphological analysis of cells

The morphological changes in human DLD-1 cell line 24, 48, and 72 hours after treatment with MTZ in concentrations of 0.1, 1, 10, 50, and 250 μM , compared with control cells, were defined by transmission electron microscope OPTON 900. The cells were prepared using the modified method presented by Hobot et al.¹⁸

Statistical analysis

The results were analyzed by Statistica 10 using ANOVA and Student's *t*-tests at a significance level of $p < 0.05$.

Results

Cell viability

Viability of cells treated 24, 48, or 72 hours with different concentrations of MTZ (0.1, 1, 10, 50, and 250 $\mu\text{g/mL}$) is shown as a percent of control (see Fig. 1).

FIG. 1. Effect of MTZ on DLD-1 cell viability presented as a percent of control. *** $p < 0.001$ versus control group without MTZ. ^^^ $p < 0.001$ versus equivalent MTZ concentration of 24 hours' group; # $p < 0.05$ versus equivalent MTZ concentration of 48 hours' group. The graph represents the viability of cells treated with MTZ in the following concentrations: 0.1, 1, 10, 50, and 250 $\mu\text{g/mL}$, after 24, 48, and 72 hours. Viable cells were determined by MTT test.

Cell viability was diminished in all experimental groups in comparison with the control, and the differences were statistically significant.

After 24-hour incubation, viability of DLD-1 cells decreased significantly compared with cells without the drug with the least viability of 86.9%, 87.8%, and 87.7% when concentrations of 10, 50, and 250 $\mu\text{g/mL}$ of MTZ were used ($p < 0.001$). We observed that the effect of MTZ (0.1, 1, and 250 $\mu\text{g/mL}$) changed in time (compared with the first time of observation) and exerted a beneficial impact due to inhibition of cell growth after 72 hours of drug treatment.

[3H]-thymidine incorporation

We found no statistically significant differences in all experimental groups and times of observation (Fig. 2).

Apoptosis and necrosis

The control group and other groups were compared in terms of their apoptosis and necrosis percentage. Data obtained with cytofluorimetric assays demonstrated a statistically significant increase of apoptotic rate in MTZ concentrations of 10 and 50 $\mu\text{g/mL}$ after 24 hours; 0.1, 10, 50, and 250 $\mu\text{g/mL}$ after 48 hours; and in all concentrations after 72 hours compared with control groups.

The percentage of necrotic cells was significantly lower than the percentage of those that underwent apoptosis. A significant increase of necrotic cells was noted after 24 hours in MTZ concentrations of 10 and 50 $\mu\text{g/mL}$. The lowest marked necrotic percentage was seen after 48 hours in 10 $\mu\text{g/mL}$ of MTZ; while after 72 hours, the percentage of necrotic cells was statistically significantly lower in MTZ concentra-

tions: 0.1, 1, 50, and 250 $\mu\text{g/mL}$ compared with the cells without the drug (Table 1).

Apoptotic and necrotic features were confirmed by fluorescence microscopy assay (Fig. 3).

DLD-1 cell parameters

The mean cell concentration, volume, and cell diameter are shown in Table 2. We found no significant differences between all MTZ concentrations and times of exposition in all studied parameters (Table 2).

The effect of MTZ on the morphological changes of DLD-1 cell line

Control cells

The cells were round or oval, with slightly irregular contours and a few short protrusions on the surface. Cells contained large, clear, oval, or round nuclei with dispersed chromatin and one or two visible nucleoli representing high electron density. Cellular organelles were localized mainly around the nucleus. Golgi apparatus, oval or rod-shaped mitochondria with clear intermembrane spaces were clearly visible. There were a few, tubular-shaped or slightly expanded rough endoplasmic reticulum and numerous ribosomes scattered in the cytoplasm. A few fibers of intermediate filaments could be seen.

Cells after MTZ

There were no significant differences compared with the control groups, considering the same exposure time. However, one can notice some differences in the structure of nuclei. In the groups with MTZ, nuclear chromatin was

FIG. 2. Effect of MTZ on [3H]-thymidine incorporation into DLD-1 cell line. The graph shows the effects of different concentrations of MTZ (0.1, 1, 10, 50, and 250 µg/mL), after 24, 48, and 72 hours of exposition to the drug on DNA synthesis presented as [3H]-thymidine incorporation into DLD-1 cell line.

regularly distributed and usually did not have clear up and any membranous structures, which are features observed in the respective time control groups. Broadening of the nuclear envelope is observed only occasionally, which was a common phenomenon in the control groups. Generally, it seemed that the structure of nuclei in the groups with metronidazole was better preserved than in the control groups. Moreover, after 72 hours, the groups exhibited greater accumulation of glycogen granules in the cytoplasm than in the control groups. In the MTZ groups, necrotic or apoptotic cells were occasionally seen (Fig. 4).

Discussion

MTZ, a synthetic derivative of nitroimidazole, is a drug that is widely used in the treatment of bacterial and anti-protozoal infections. MTZ is active against most obligate

anaerobes but does not appear to have any activity against facultative anaerobes or obligate aerobes.¹⁹ Some chemotherapeutic agents used in cancer treatment may contribute to a secondary tumor by interacting with DNA. Interaction of MTZ's metabolites with DNA led to the drug examination for possible effectiveness of radiation therapy in cancer patients.²⁰ In animal experiments, the administration of MTZ resulted in malignant lymphomas and lung tumors in mice, hepatoma and breast tumors in female rats, and tumors of Leydig cells and pituitary adenoma in male rats.^{11,20,21} Nevertheless, carcinogenicity data on humans are inconclusive. MTZ's effects, besides those commonly known, were also studied in cell cultures.

DLD-1 is one of two colorectal adenocarcinoma cell lines that were isolated by D.L. Dexter and associates during a period from 1977 to 1979 (ATTC). Our results on DLD-1 cells show the beneficial effect of MTZ after even a 24 hour

TABLE 1. APOPTOSIS AND NECROSIS OF DLD-1 CELL LINE TREATED WITH METRONIDAZOLE

		MTZ					
		Control	0.1 µg/mL	1 µg/mL	10 µg/mL	50 µg/mL	250 µg/mL
Apoptosis (%)	Time						
	24h	8.98 ± 0.61	8.56 ± 1.66	8.98 ± 0.56	12.24 ± 1.59 ^a	14.34 ± 1.58 ^b	11.22 ± 2.11
	48h	3.53 ± 0.57	6.9 ± 1.71 ^a	5.2 ± 1.74	10.13 ± 1.59 ^a	9.26 ± 0.73 ^b	13.2 ± 4.40 ^c
Necrosis (%)	Time						
	24h	0.8 ± 0.29	0.86 ± 0.18	0.675 ± 0.22	2.675 ± 1.23 ^c	2.9 ± 0.78 ^a	1.52 ± 0.58
	48h	1.93 ± 0.15	4.125 ± 1.47	2.56 ± 1.28	1.64 ± 0.15 ^c	2.22 ± 0.58	2.18 ± 0.44
	72h	4.86 ± 4.07	4.76 ± 0.51 ^c	4.00 ± 0.91 ^c	6.17 ± 0.29	1.66 ± 0.57 ^b	3.32 ± 1.52 ^a

^a*p* < 0.01.

^b*p* < 0.001 versus control group.

^c*p* < 0.05.

MTZ, metronidazole.

FIG. 3. Fluorescence microscopy of DLD-1 cell line treated with MTZ. Photomicrographs of DLD-1 cell line stained with acridine orange and ethidium bromide. Cells treated for: 24 hours with 50 µg/mL MTZ (A), 48 hours with 250 µg/mL MTZ (B), 72 hours with 50 µg/mL MTZ (C), and control cells (D) after 72 hours (magnification $\times 400$). Apoptotic cells were observed in treated samples.

incubation, as we received a statistically significant increase of cytotoxicity (10, 50, and 250 µg/mL). According to our observation, one dose of the drug is enough to reduce the number of viable cells. Moreover, it seems that the longer the exposition to the drug occurs, the better anticancer effect it can cause.

Similar results were obtained by Ferreira et al.²² Authors presented time- and concentration-dependent cytotoxicity of antibiotics used in endodontic therapy. All drugs (ciprofloxacin, clindamycin, and MTZ) tested on human gingival fibroblasts showed dose-dependent cytotoxicity. MTZ was used in the following concentrations: 5, 50, 150, and 300 mg/L for 24, 48, 72, and 96 hours. All concentrations of the drug led to at least 50% viable cells in all experimental times. Compared with other drugs, MTZ presented the highest viability at 72 and 96 hours.²²

On the contrary, Fargue-Lafitte et al.²³ acquired some different results. They tested the growth-inhibitory effects of

ketoconazole in human colon and breast cancer cell lines and the proliferation of HT29-S-B6 cells in the presence of several substituted azole derivatives, used as antifungal or antibacterial agents. MTZ, secnidazole, and also other azole derivatives that do not inhibit cytochrome P-450-dependent enzymes had no growth-inhibitory activity (10–100 µM).²³

Chinese hamster ovary cells (CHO-kl) were grown to evaluate the cytotoxic activity of MTZ (Flagyl). Mahood and Willson²⁴ showed that Flagyl (5 mM) was only cytotoxic at high initial densities (5×10^6 /mL) in ampoules. The percentage cell viability dropped to 0.3% in 8 hours. Experiments repeated in spinner flasks with an initial cell density of 5×10^4 /mL and the drug showed little toxicity, with the percentage cell viability dropping to only 65% after 8 hours.²⁴

MTZ has also been proposed in the field of periodontal therapy either with a systemic administration, mostly in combination with amoxicillin or ciprofloxacin, or with local

TABLE 2. EFFECTS OF METRONIDAZOLE ON DLD-1 CELL PARAMETERS (CONCENTRATION, VOLUME, AND DIAMETER)

	Time	MTZ					
		Control	0.1 µg/mL	1 µg/mL	10 µg/mL	50 µg/mL	250 µg/mL
Concentration (cells/mL $\times 10^5$)	24 h	1.842 $\pm$ 0.03	2.718 $\pm$ 0.03	2.20 $\pm$ 0.27	2.103 $\pm$ 0.23	2.357 $\pm$ 0.51	2.922 $\pm$ 0.09
	48 h	2.456 $\pm$ 0.30	2.756 $\pm$ 0.30	2.015 $\pm$ 0.09	2.541 $\pm$ 0.13	2.213 $\pm$ 0.02	2.417 $\pm$ 0.24
	72 h	3.521 $\pm$ 0.09	3.012 $\pm$ 0.03	3.419 $\pm$ 0.33	3.427 $\pm$ 0.24	2.971 $\pm$ 0.05	3.373 $\pm$ 0.26
Volume (pL)	24 h	4.581 $\pm$ 0.128	5.00 $\pm$ 0.17	3.30 $\pm$ 0.42	5.429 $\pm$ 0.32	5.866 $\pm$ 0.08	4.836 $\pm$ 0.06
	48 h	3.526 $\pm$ 0.65	3.055 $\pm$ 0.82	2.89 $\pm$ 0.11	3.272 $\pm$ 0.39	5.267 $\pm$ 0.41	4.859 $\pm$ 0.17
	72 h	2.673 $\pm$ 0.2	2.958 $\pm$ 0.07	4.855 $\pm$ 0.74	3.141 $\pm$ 0.31	6.023 $\pm$ 0.46	2.163 $\pm$ 0.21
Diameter (µm)	24 h	20.79 $\pm$ 0.29	20.395 $\pm$ 0.26	19.985 $\pm$ 0.04	21.651 $\pm$ 0.63	22.55 $\pm$ 0.35	20.599 $\pm$ 0.62
	48 h	18.545 $\pm$ 0.74	17.925 $\pm$ 1.62	17.665 $\pm$ 0.22	18.40 $\pm$ 0.75	20.975 $\pm$ 1.41	18.375 $\pm$ 3.47
	72 h	17.205 $\pm$ 0.44	18.775 $\pm$ 1.52	18.775 $\pm$ 1.07	20.975 $\pm$ 0.60	22.11 $\pm$ 1.22	23.016 $\pm$ 1.42

FIG. 4. Effect of MTZ on the morphological changes of DLD-1 cell line. Figure represents electron micrographs of DLD-1 cancer cell lines. **(A)** 250 $\mu\text{g}/\text{mL}$ of MTZ after 72 hours; arrow points to necrotic cell (magnification $\times 4400$). **(B)** 250 $\mu\text{g}/\text{mL}$ of MTZ after 72 hours; arrow points to apoptotic body (magnification $\times 4400$). **(C)** Control cells (without MTZ) after 24 hours (magnification $\times 4400$). **(D)** Control cells (without MTZ) after 72 hours (magnification $\times 3000$).

biodegradable sustained-release agents. The immunomodulatory effects of antibiotics might influence the degree of the local response to infection in the human periodontal ligament cell (HPLC). HPLCs play a role in the immune response of the oral cavity. Therefore, the aim of the study of Rizzo et al.²⁵ was to simulate the *in vivo* conditions occurring in diseased periodontal sites, and to evaluate the effects of MTZ on the viability of isolated HPLCs.²⁵ For cell proliferation experiments, the HPLC cultures were incubated with MTZ (2.5, 25, 250, and 2500 $\mu\text{g}/\text{mL}$) at 37°C in CO_2 for 24, 48, and 72 hours. The analysis of the effect of MTZ (2.5, 25, 250, and 2500 $\mu\text{g}/\text{mL}$) on the proliferative activity and viability of HPLCs shows that the untreated HPLC did not show any statistical difference in terms of cytotoxicity, cell counts at the different times. Likewise, the cytotoxicity index of the cells treated with MTZ ranged only from 7% to 29%, compared with the control. The effect of MTZ on HPLC was not cytotoxic at any of the study times; whereas at a concentration of 2500 $\mu\text{g}/\text{mL}$, the cytotoxicity index was greater than 50%.²⁵

MTZ was evaluated as a single agent or in the combination with CDDP (cisplatin) and araC (cytosine arabinoside) for its cytotoxic effects on five human colon carcinoma cell lines. MTZ alone produced little cytotoxicity at 1 hour incubation, and it was detectable only after 2 hours of incubation and increased as a function of duration of treatment, which suggested a time-dependent rather than a dose-dependent cytotoxic effect. MTZ had no effect on CDDP- or

araC-induced cytotoxicity, but enhanced the synergism resulting from the combination of two antitumor agents on the tested cell lines.²⁶

MTZ has been applied as a radiosensitizer of hypoxic or tumor cells in order to increase the cytotoxic effects of certain antitumor agents. Other studies were performed to assess whether MTZ enhances the cytotoxicity of 5-fluorouracil (5-FU).²⁷ Trying to simulate conditions observed in patients, HCT-8 colon cancer cell lines were exposed to 100 $\mu\text{g}/\text{mL}$ MTZ for 1 or 24 hours under aerobic conditions, followed by 1 hour incubation with 5-FU. MTZ did not modify the cytotoxicity of 5-FU. In other set of experiments, cells were incubated for 10 days with both MTZ (50 $\mu\text{g}/\text{mL}$) and 5-FU, and this treatment also did not show any enhancement of cytotoxicity by MTZ. Again, 2-hour incubation of HCT-8 cells with MTZ (5 mg/mL) in hypoxic conditions with either 1 hour or 10 day exposure to 5-FU also did not reveal any modification of 5-FU cytotoxic effects. Moreover, control incubation of colon cancer cells with MTZ alone (5 mg/mL for 2 hours or 100 $\mu\text{g}/\text{mL}$ for 24 hours) under aerobic conditions or under hypoxic conditions (5 mg/mL for 2 hours) had no effect on cell viability.²⁷

Mohindra and Rauth²⁸ studied the effects of MTZ and nitrofurazone on cell viability *in vitro* in the presence of air or nitrogen without radiation. The toxicity of different concentrations of drugs was measured by incubating the cells continuously in the drug-containing medium. All of the cells:

CHO, HeLa (derived from cervical cancer), and human marrow cells responded similarly to increasing concentrations of MTZ and nitrofurazone in the medium. The concentration of MTZ that inhibited colony-forming ability by 50% was 10 mM. This result indicates that for assay cell survival, after exposure to a high concentration of the drug, it has to be reduced below 1 mM to avoid residual toxicity. After 8 hours of incubation of Chinese hamsters ovary cells in 29 mM MTZ, the absolute plating efficiency remained relatively constant in the presence of air (80%–40%). On the contrary, under hypoxic conditions, the plating efficiency of the cells dropped to 1% after 6 hours of incubation in 29 mM of MTZ.²⁸

MTZ and its hydroxy metabolite (MTZOH) are compounds that have the capacity to produce damages in macromolecules such as DNA, which is the basic mechanism of action in bacteria. It has been shown that MTZ is intracellularly activated by reduction, and the toxic effect of reduced intermediates bind to DNA, leading to loss of helical structure, strand breakage, and impairment of DNA function.²⁹

Our results on ³[H]-thymidine incorporation into DNA indicate that MTZ did not cause any significant changes in all times of exposition. Differences between cell survival and DNA biosynthesis could be explained as the defense against cell death and immediate compensation presented as thymidine incorporation.

Since the nitroheterocycles inhibit the incorporation of ³[H]-thymidine by L-929 cells (mouse fibroblast cells) cultured under aerobic conditions, Olive³⁰ selected three of them, including MTZ, for detailed studies of the mechanism of inhibition of DNA synthesis. The incubation with MTZ for 1 hour under aerobic conditions produced marked inhibition of DNA-precursor incorporation. There were no significant effects on DNA damage, elongation, or cell survival. After 4 hours of incubation under anaerobic conditions, all endpoints indicated damage to L cells. The rate of incorporation of ³[H]-thymidine did not increase after drug treatment for at least 4 hours.³⁰ These results are similar to our observation after 24 hours. Probably such time of observation is the time after the drug already affected the DNA, and longer exposition is irrelevant as cell proliferation remains unchanged.

Both MTZ and MTZOH are able to increase cell proliferation, although this mechanism is not well understood. Bahr and Ullmann³¹ performed a study on the immunomodulating properties of MTZ and its metabolites. MTZ and its hydroxy metabolite enhanced the mitogenic stimulation of murine lymphocytes. Maximum enhancement was in the same concentration range (32 and 64 μ g/mL) and resulted in a 7.4-fold increase for MTZ and a 40.5-fold (hydroxy metabolite) increase of (3H)-thymidine incorporation compared with the control.³¹ In addition, Menéndez et al.²⁹ showed that MTZOH is able to induce the expression of P53, which has earlier been proposed as a biomarker of carcinogenicity.²⁹ Studies on P53 functionality in the genotoxicity of MTZ and MTZOH led authors to interesting conclusions. Cell proliferation was determined on a few cell lines, including human cervical carcinoma cells (HeLa) and colon carcinoma cell lines (RKO and RKO-E6). They observed an increase in cell proliferation in all cell lines treated with MTZ, irrespective of their P53 functionality; whereas MTZOH only induced an increased proliferation in cell lines with abnormal P53.²⁹

In the present work, we show apoptotic and necrotic responses to MTZ stimuli. Apoptosis is characterized by morphological changes in nucleus and cytoplasm. This includes shrinkage of the cell, increasing permeability of membrane, chromatin condensation, and externalization of plasma membrane (PS).³²

Incubation with PI was used to investigate membrane integrity in cells undergoing apoptosis, as PI can only enter cells with altered plasma membrane and give red fluorescence. This dye is used along with Annexin V, which binds PS on the plasma membrane during apoptosis. Flow cytometry data shown increased cell permeability due to MTZ treatment. The results were statistically significant in all experimental periods. Annexin V binding was the highest in cells treated with 250 μ g/mL of MTZ, especially after 72 hours. It is worthy of note that there were also some statistical changes in PI binding compared with the control. A significant increase in necrotic cells was noted after 24 hours in MTZ concentrations of 10, 50, and 250 μ g/mL and after 48 hours in the lowest concentrations (0.1, 1 μ g/mL). The lowest marked necrotic percentage was seen after 48 hours in 10 μ g/mL of MTZ; while after 72 hours, the percentage of necrotic cells was statistically significantly lower in MTZ concentrations of 0.1, 1, 50, and 250 μ g/mL compared with the cells without the drug. Cells treated with MTZ, after staining with acridine orange, revealed characteristic apoptotic features such as shrinkage and chromatin condensation, supporting our results from flow cytometry assay. These results are consistent with data obtained by MTT test presenting cell viability, which is in agreement with a statement that the loss of cell viability is often measured as loss of membrane integrity. Unfortunately, there is lack of data in the literature dedicated to the influence of MTZ on the apoptotic process in cell lines. However, MTZ has been shown to induce programmed cell death in the protozoan parasite *Blastocystis hominis*. The drug is used clinically to treat infections caused by this intestinal parasite. Authors demonstrated characteristic features of programmed cell death of *B. hominis* after exposure to 5×10^{-7} M MTZ. Light microscopic data showed shrinkage and darkening of the cytoplasm in *B. hominis*, which was supported by flow cytometry data. In addition, transmission electron micrographs of MTZ-treated parasite also showed ultrastructural characteristics of apoptosis.³³

In conclusion, MTZ reduced cell viability of DLD-1, which was consistent with the apoptotic assay.

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Disclosure Statement

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