The relationship between metronidazole concentration and clinicopathological parameters in patients with colon cancer: A pilot study

Sadowska A.[1], Car H.[1*], Rodziewicz L.[2], Cepowicz D.[3], Kędra B.[3]

[1] Department of Experimental Pharmacology, Medical University of Białystok, Poland
[2] Department of Hygiene Veterinary, Białystok, Poland
[3] 2nd Department of General and Gastroenterological Surgery, Medical University of Białystok, Poland

ABSTRACT

Purpose: To evaluate the concentration of metronidazole (MTZ) and its hydroxy metabolite (MTZOH) in cancer tissue and adjacent normal tissue in colorectal cancer patients in correlation with clinicopathologic parameters.
Material and methods: MTZ and MTZOH concentration were measured in tumor tissue and surrounding healthy tissue by LC-ESI-MS-MS method.
Results: We found different concentration of MTZ and MTZOH in colorectal cancer and healthy tissue, however the results were not statistically significant. MTZ concentration was elevated in tumors located in rectum, in patients over 60 years old, in patients without metastases to regional lymph nodes (N0) while decreasing with increasing tumor size. Women accumulated greater amounts of MTZ in comparison to men.
Conclusion: Comparison of the concentration of the drug and its metabolite in tumor and normal colon tissue shows its different reaction to MTZ. MTZ concentration in the tumor and normal colon tissue is sex-dependent.
Key words: Metronidazole, hydroxy metabolite, colorectal cancer, colon healthy tissue

***Corresponding author:**
Department of Experimental Pharmacology
Medical University of Bialystok
Szpitalna 37, 15-295 Bialystok, Poland
Tel: +48 857485554, Fax: +48 857485554
e-mail: hcar@umb.edu.pl

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INTRODUCTION

Metronidazole (MTZ) is an antimicrobial agent that has been used in medicine for over 45 years. The drug is effective for the management of anaerobic infections, such as intra-abdominal and gynecologic infections, septicemia, endocarditis, bone and joint infections, central nervous system infections, respiratory tract infections, skin, and oral and dental infections. Moreover, MTZ is applied for treatment of giardiasis, trichomoniasis and amoebiasis, and it is recommended for the treatment of patients with bacterial vaginosis or vaginitis caused by *Gardnerella vaginalis*. Metronidazole is also used as prophylaxis before abdominal and gynecological surgery to reduce the risk of postoperative anaerobic infection.

Antimicrobial prophylaxis is indicated particularly where anaerobic bacteria predominate, which is in the surgery of large intestine and rectum [1,2]. High risk of perioperative infections is associated with tumor resection in colorectal cancer patients. The use of metronidazole 500 mg infused intravenously at the beginning of the operation, either with or without an antibiotic against aerobic bacteria, has reduced the wound infection rate to between 7% and 20% [3]. Interaction of metronidazole metabolites with DNA led to the examination of metronidazole for possible efficacy of irradiation in cancer patients [4]. Experiments on animals revealed that metronidazole caused lymphomas and lung tumors in mice, hepatomas and breast tumors in female rats and Leydig cell tumors and pituitary adenomas in male rats. However, in most experiments MTZ dosages were relatively higher than those used to treat infections in humans [4-6]. Tumor induction in animals tends to reflect on MTZ action, especially due to its common use in the prevention of perioperative infections in patients before surgery of gastrointestinal cancer.

The aim of our study was to evaluate the concentration of MTZ and its hydroxy metabolite in healthy and cancerous colon tissue in correlation with clinicopathologic parameters.

MATERIALS AND METHODS

Patients

The study was performed on colorectal resection specimens removed from cancer and from healthy tissue. The tissues were obtained from 30 patients: 16 women and 14 men aged 25 to 85 years (mean 63±15.7). Patients were diagnosed and referred for surgical resection of colorectal cancer in the 2nd Clinical Department of General and Gastroenterological Surgery at the University Hospital in Białystok. Colorectal cancer diagnosis was based on clinical symptoms, colonoscopy and histopathological analysis of tumor and surrounding lymph nodes taken during surgery. All patients were classified histopathologically as adenocarcinoma and represented tumors in pT3 stage. According to Dukes classification: 1 patient was included into A class, 13 into B, 10 into C and 6 into D class. 14 patients had metastases to regional lymph nodes (N+) (Table 1).

The study protocol was approved by the Ethic Committee of Medical University of Bialystok. Each subject gave written informed consent for participation in the study.

Drug - All patients had been given 500 mg of the metronidazole iv 2 h prior to surgery.

Tissue samples - Tumor samples and healthy colon tissue, that were about 10 cm away from cancer, were obtained about 4–5 h after administration of MTZ. The material was collected during surgery and frozen immediately in liquid nitrogen, and then stored at -80°C until examination.

LC-ESI-MS-MS method of MTZ and MTZOH evaluation

Chemicals and regents - Metronidazole (MTZ), its hydroxy metabolite (MTZOH) and deuterated internal standard (IS) hydroxymethylnitroimidazole d3 (HMMNI d3) were obtained from Riedel-de-Haën (Fluka, Buchs, Switzerland). Acetonitrile, methanol LC-MS, methanol LC, acetonitrile LC, acetone formic acid (80–100%) were purchased from Merck (Darmstadt, Germany). Acetic acid (95.5%), sodium sulfate, ammonia solution (25%) were provided by Baker (Deventer, The Netherlands). Strata SCX (500 mg/3 ml) solid phase extraction (SPE) cartridges were supplied by Phenomenex (Cat. No. 8B-SO10-HBJ).

Standard solutions were prepared according to the method described by Mottier et al. [7].

Sample preparation - The sample was weighed into glass tube and then internal standard and acetonitrile were added. After sodium sulfate adding, the sample was centrifuged at 3500 × g for 10 min. The supernatant was transferred into a flask through 15 g of sodium sulfate and 5 ml of acetic acid was added. The extract was cleaned up on SCX column and column was conditioned with 5% acetic acid in acetonitrile, washed with acetone, methanol and acetonitrile, and finally, dried for 10 min. The nitroimidazoles were then eluted with 5% ammonia in acetonitrile. The sample was evaporated to dryness under a nitrogen stream.

Table 1. Clinicopathologic characteristic of study group.

Parameter		No. of patients N=30 (100%)
Age	≤60 >60	13 (43.3%) 17 (56.7%)
Gender	W M	16 (53.3%) 14 (46.7%)
Location	colon rectum	21 (70%) 9 (30%)
Histologic type	Adenocarcinoma	30 (100%)
Histologic grade (G)	G2 (moderately) G3 (poorly differentiated)	27 (90%) 3 (10%)
Lymph node involvement (pN)	N0 N+	16 (53.3%) 14 (46.7%)
Dukes classification	A B C D	1 (3.4%) 13 (43.3%) 10 (33.3%) 6 (20%)
Size of the primary tumor	<5 cm >5 cm	23 (85%) 4 (15%)

LC-ESI-MS-MS - LC analyses were performed on a Luna C18(2) column (150 x 2 mm i.d., 3 μm) (Phenomenex, Torrance, USA) using an Agilent 1100 series liquid chromatography equipped with a binary pump and an autosampler (Agilent Technologies, Waldbronn, Germany). MS analyses were carried out on an API 4000 triple stage quadruple mass spectrometer (Applied Biosystems, Foster City, CA, Canada) equipped with a turbo ion spray interface. Detailed LC-ESI-MS-MS method was described in our previous study [8].

Statistical analysis

The data are presented as the means ± SD. The statistical significance of the differences between the means was determined by PQStat ver. 1.4.2.324 using U Mann-Whitney test; $p < 0.05$ was considered as the level of significance.

RESULTS

The concentration of MTZ and MTZOH in tumors equaled 3.05±2.07 and 0.61±0.68 μg/g tissue, while in healthy tissue: 2.75±1.26 and 0.55±0.49 μg/g tissue, respectively. The differences of results did not differ significantly. The level of MTZ and MTZOH in tumors of patients with metastases to regional lymph nodes (N+) was slightly decreased in comparison to healthy tissue. On the contrary, in N0 cancer, both MTZ and MTZOH levels were not markedly elevated in tumors vs. normal colon tissues. When we compared the amount of the drug and its metabolite in metastatic and non-metastatic cancer, it turned out that higher MTZ and MTZOH concentrations occurred in N0 tissues. In normal colon tissue the results were similar in both groups of patients, and the results did not reach statistical significance (Table 2).

Table 2. Concentration of MTZ and MTZOH in tumor and healthy tissue of CRC patients.

	N0		N+	
	MTZ (μg/g tissue)	**MTZOH (μg/g tissue)**	**MTZ (μg/g tissue)**	**MTZOH (μg/g tissue)**
tumor	3.92±3.3	0.78±0.8	2.09±1.2	0.31±0.4
healthy tissue	2.95±1.3	0.62±0.3	2.21±1.7	0.41±0.6

MTZ-metronidazole
MTZOH- metronidazole hydroxy metabolite
N0- patients without metastases
N+ - patients with metastases to regional lymph nodes

Increased concentrations of MTZ in tumors of men and women comparing to adjacent healthy tissues were without significance. The content of the drug was higher in female group, both in healthy and tumor tissues, when compared to male patients and these results were statistically significant (Table 3). The concentration of MTZOH was higher in tumor and healthy tissues of women, but not reaching statistical significance.

The mean concentrations of MTZ and its metabolite in tumor of patients under 60 years old equaled 2.93±1.6 and 0.12±0.07 μg/g tissue respectively, while in patients over 60 were higher

and equaled 4.29±2.1 and 0.68±0.72 μg/g tissue. The differences of results were not statistically significant (Table 4).

Table 3. Concentration of MTZ and MTZOH in tumor and healthy tissue of CRC patients.

	Men		Women	
	MTZ (μg/g tissue)	**MTZOH (μg/g tissue)**	**MTZ (μg/g tissue)**	**MTZOH (μg/g tissue)**
tumor	2.77±1.1	0.12±0.1	4.42±1.6*	0.94±0.8
healthy tissue	1.61±0.4	0.2±0.1	4.25±0.7***	0.8±0.5

*p<0.05; ***p<0.001 men (MTZ) vs women (MTZ)
MTZ-metronidazole
MTZOH- metronidazole hydroxy metabolite

Table 4. Concentration of MTZ and MTZOH in tumor and healthy tissue of CRC patients according to age.

	≤60		>60	
	MTZ (μg/g tissue)	**MTZOH (μg/g tissue)**	**MTZ (μg/g tissue)**	**MTZOH (μg/g tissue)**
tumor	2.93±1.6	0.12±0.07	4.29±2.1	0.68±0.7
healthy tissue	1.71±0.6	0.25±0.01	3.39±1.4	0.57±0.6

MTZ- metronidazole
MTZOH- metronidazole hydroxy metabolite

Higher concentration of MTZ and MTZOH in tumors with diameter smaller than 5 cm and surrounding normal tissue comparing with larger tumors (> 5 cm) and healthy tissue were not statistically significant (Table 5).

Table 5. Concentration of MTZ and MTZOH in tumor and healthy tissue of CRC patients according to the size of primary tumor.

	<5 cm		>5 cm	
	MTZ (μg/g tissue)	**MTZOH (μg/g tissue)**	**MTZ (μg/g tissue)**	**MTZOH (μg/g tissue)**
tumor	3.67±2.2	0.59±0.7	2.9±0.28	0.21±0.0
healthy tissue	3.12±1.5	0.53±0.5	1.93±0.0	0.2±0.00

MTZ-metronidazole
MTZOH- metronidazole hydroxy metabolite

Analysis of MTZ concentration depending on the location of primary tumor showed its higher levels in tumors and healthy tissues of rectum, while MTZOH concentrations were elevated in other parts of the colon (Table 6).

Table 6. Concentration of MTZ and MTZOH in tumor and healthy tissue of CRC patients according to location of the primary tumor.

	Colon		Rectum	
	MTZ (μg/g tissue)	**MTZOH (μg/g tissue)**	**MTZ (μg/g tissue)**	**MTZOH (μg/g tissue)**
tissue	3.28±1.2	0.55±0.7	4.14±2.2	0.25±0.1
healthy tissue	2.62±1.04	0.44±0.3	4.51±0.3	0.04±0.0

MTZ-metronidazole
MTZOH- metronidazole hydroxy metabolite

The assessment of MTZ and its metabolite was performed in cancer tissues in regards to Dukes classification. Patients were classified to B, C and D class according to above classification. MTZ and MTZOH amounts were the highest in colon tissues (tumor and healthy surrounding) of patients from B class and the lowest in D class (Table 7). The differences of results were not obtained statistically significant level.

Table 7. Concentration of MTZ and MTZOH in tumor and healthy tissue of CRC patients according to Dukes classification.

	B		C		D	
	MTZ (μg/g tissue)	**MTZOH (μg/g tissue)**	**MTZ (μg/g tissue)**	**MTZOH (μg/g tissue)**	**MTZ (μg/g tissue)**	**MTZOH (μg/g tissue)**
tumor	4.41±2.61	0.85±0.96	3.47±1.55	0.50±0.55	3.08±1.89	0.14±0.09
healthy tissue	2.96±1.54	0.63±0.39	3.61±1.90	0.53±0.78	2.05±0.16	0.23±0.03

MTZ-metronidazole
MTZOH- metronidazole hydroxy metabolite
B,C,D – Dukes' classification stages

DISCUSSION

Improvement of surgical techniques and the use of broad spectrum antibiotics and chemotherapeutic e.g., metronidazole, changed the view of colorectal surgery. However, it is still a big challenge, especially in the elderly and affected by cardiovascular diseases. Big problem is produced by septic complications, which occur in 7-23% of patients operated during elective surgery [9]. Many studies have shown that perioperative antibiotic prophylaxis (PAP) reduced the incidence of postoperative septic complications [10 -12]. PAP is achieved with an antibiotic treatment before surgery to destroy microorganisms from both, the environment and patients own flora, which are a potential source of infection after surgery [13]. Prevention of postoperative septic complications in colon surgery should begin shortly before the surgery, and last from 24 to 72 hours, so as to ensure that the natural defense mechanisms are effective during operation. As the bacterial flora of intestine consists mainly of obligate anaerobes, metronidazole has become the drug of choice against anaerobes, significantly reducing the incidence of postoperative infection [14]. Metronidazole is a drug widely used, and its availability is facilitated by the fact that it can be administered by various routes: topical, oral, intravenous, rectal and vaginal. Furthermore, MTZ penetrates to all tissues, it can also cross blood-brain barrier and the seminal fluid [15,16].

In the present study we have selected patients with colorectal cancer (CRC) diagnosed as adenocarcinoma. On the basis of our results and histopathological examination, patients were divided according to parameters such as gender, age, location, tumor size, histological type, degree of tumor differentiation, and Dukes classification. Among 30 patients included in the study, 16 were women and 14 men. Patients were also divided according to age – under 60 and over 60 years old. Considering the location of the primary tumor, we have distinguished two groups: first had neoplasms located in the anus and rectum, whereas the second for simplicity includes tumors from other parts of the intestine such as the cecum, ascending colon, sigmoid colon, hepatic flexure and descending colon. Tumors were also divided depending on the size of their diameter. The first group included 23 tumors ranged from 2 to 5 cm, while 4 tumors measured more than 5 cm. The concentration of metronidazole and its major metabolite was investigated in tumors and macroscopically unchanged tissue, adjacent to the tumor taken during a bowel resection.

The mean concentration of MTZ in tumor equaled 3.05±2.07 μg/g tissue and was higher but not statistically significant than in normal colon tissue (2.75±1.26 μg/g tissue). Similar results were obtained in our previous study. We also observed the tendency to MTZ accumulation in tumors [8]. In the scientific literature there are only few studies that have specifically examined the concentration of metronidazole in patients undergoing colorectal surgery. One study evaluated the concentration of MTZ in the straight abdominal muscle and colon mucosa. 12 patients undergoing colorectal surgery received intravenously 1 g MTZ as a prophylaxis. The mean ratios of tissue:serum was 0.94 and 0.76, respectively [17]. Similar studies conducted Martin et al [18], who found that the ratio of abdominal wall fat to the plasma was 0.1, while the average concentration of MTZ in intestinal tissue equaled 8.9 mg/kg [18].

Bergan et al. [19] after intravenous administration of 1.5 g MTZ marked concentration of the drug and its metabolite in various tissues such as: in the colon, peritonitis, ileum, and appendix. Mean concentration of MTZ in various tissues ranged from 10 to 30 mg/g, while the hydroxy metabolite concentration was lower than 5 mg/g. Metronidazole was detectable in tissues after 48 hours, while its metabolite - after 72 hours, which indicates that metabolite also acting on the anaerobic bacteria, extended the duration of the biological activity of the drug [19]. The differences between our results and those in the literature may be due to different doses applied to patients in the current study, lower than those, used in the studies of other authors. According to Bergan et al [19], material for the determination of MTZ and its metabolite was collected 48 and 72 h after administration, which shows that the time to excise biological samples was much longer in comparison to the present study - up to 5 hours after drug dosing. Moreover, the method used here for the assessment of the drug and its metabolite is modern and it was not available in the past decades.

Determination of metronidazole and its hydroxy metabolite showed that primary tumors that metastasize to regional lymph nodes (N+) are characterized by a twice lower concentration of the drug compared to tumors non-metastatic (N0). Possibly reduced, both the absorption and accumulation of the drug in the primary tumor suggests that MTZ penetrates to the metastatic tumor lesions in the lymph nodes, which is associated with EPR (enhanced permeability and retention) effect.

We observed higher concentrations of MTZ and MTZOH in tumor than in normal surrounding tissue, but it did not reach statistical significance. Differences in concentration might be due to the increased absorption and accumulation of the drug in tumor. The above pharmacokinetic parameters of MTZ can depend on the size of the tumor, its morphological structure, or the age and gender of patients.

Considering Dukes classification, we observed that MTZ concentrations in B and D classes were higher in tumors compared to healthy tissue. The above results in B class are interesting and confirm the observation of higher MTZ concentrations in non-metastatic tumors. However, surprisingly higher MTZ concentrations in D class requires further study to determine the mechanism of the pharmacokinetics of this chemotherapeutic agent.

Comparing the drug concentration in various sizes of tumor resulted the observation that the highest average concentration of MTZ and its metabolite (MTZOH) are located in tumors with the smallest diameter. Furthermore, an increased concentration of the drug and its metabolite in tumor tissue compared to normal tissue was observed. The differences were not statistically significant, and perhaps mean drug concentrations are due to different number of tumors in various sizes. This phenomenon is even more difficult to explain, since it can be assumed that larger tumors, in relation with increased hypoxia should absorb larger amounts of the drug. This could elucidate the tendency for a higher concentration of MTZ and its metabolite in the tumor tissue compared to a healthy one. Moreover, these results could be explained on the basis of the EPR effect, which was applied to optical imaging and targeting of the tumor. The effect of enhanced permeability and retention was first described by Matsumura and Maeda in 1986 [20]. The process of angiogenesis is promoted from the earliest stages of carcinogenesis, in order to provide required supplies of nutrients and oxygen to the rapid growth of the tumor. However, the newly formed tumor vessels are usually disturbed structurally, which results in their increased permeability. In addition, the lack of effective lymphatic drainage leads to impaired growth and fluid transport molecules, resulting in selective leakage of macromolecules from tumor vessels and their accumulation in tumor parenchymal cells [20-23].

Another aspect is the difference in the concentration of MTZ in tumors depending on the age of patients. The World Health Organization (WHO) divides the elderly people into several groups. People from 45 to 60 years old people are defined as aging, and from 61 to 75 years - elderly people. Therefore, patients in our study were divided into two groups: under 60 and over 60 years old. In this study, the correlation between age and MTZ concentration showed almost 1.5-fold higher concentration of the drug in people over 60 years old, as well as significantly higher metabolite concentrations in these patients. In addition, in healthy tissue the concentration of both MTZ and MTZOH was 2-fold higher in older group of patients. Comparison of these parameters in normal and tumor tissue showed greater concentration of tested substances in the tumor. This is probably due to the previously discussed effect of increased permeability and retention. It is known, that depending on the age, biological differences occur in the human body. Elderly patients have declining cardiovascular functionality, but also the liver and kidneys function. Furthermore, the concentration of serum albumin is decreased, which results in an increase of the drug free fraction. In addition, the activity of enzymes involved in the first phase processes (mainly oxidation), by which metronidazole is metabolized in the liver, is diminished.

The above results are important from a clinical point of view, as MTZ has been classified by the International Agency for Research on Cancer (IARC) as an animal carcinogen [24]. Rustia and Shubik [25] observation of the induction of tumors in mice and rats caused by MTZ led to speculation on its potentially carcinogenic effect. The authors found a significant increase of lung tumors and lymphomas in female mice. In addition, female rats developed hepatomas and breast tumors, whereas males showed an increase in the incidence of tumors of the pituitary gland and testes [4,26]. Other studies on the carcinogenic potential of MTZ in animals have been performed by Chacko and Bhide [5]. The drug was administered intragastrically to males and females. They observed ovarian cysts, lung, spleen, liver, and thymus tumors in 13 out of 40 females. After MTZ treatment in males, 8 out of 38 developed lung and liver tumors [5]. Based on above experiments, one can conclude that females develop tumors more frequently than males.

In the present study, similarly to our previous observation [8], women represent more than half of the population and the concentration of MTZ, both in normal and cancerous tissues was about two times higher than in men. The question is, what caused this difference, considering that the age of the majority of patients excludes the impact of hormones. The pharmacokinetics of MTZ has been investigated many times, however it does not allow us to determine the reason of the differences in concentrations between men and women. MTZ is metabolized in the liver by the cytochrome P-450. It is not known whether the liver, fulfilling a number of functions in the body is regulated differently in men and women, and whether any of its dysfunction leads to reduced clearance of the drug. Moreover, both the differences in drug metabolism as well as in protein binding may be observed in both genders, contributing to observed differences in MTZ concentrations. An interesting observation is the finding of a lower MTZOH concentration in the tumor of men. On the basis of this survey and the lack of literature data, we cannot explain the mechanism of this result.

Referring to the hormonal balance, Sohrabi et al [27] published a study showing the inhibitory effect of MTZ on spermatogenesis and sex hormones in rats. 18 Wistar rats were divided into 3 groups, first – control was treated with water, whereas the second and third were given MTZ at doses of 200 and 400 mg/kg/day for 60 days. The second and the third group revealed a significant reduction in weight of the testes and other reproductive organs, and also decreased concentration of LH, FSH and testosterone levels in the blood plasma with a massive degradation of all reproductive cells at the stage VII of the seminiferous epithelium cycle [27].

Nevertheless, data on the carcinogenicity of metronidazole in humans is inconclusive. Several epidemiological studies have been performed to determine the relationship between cancer in humans and the use of MTZ. The incidence of cervical cancer in women treated with MTZ because of trichomoniasis was determined. Although *T. vaginal* infection itself is considered as a risk factor for cervical cancer [28,29]. However, Falagas et al. [6] set out the incidence of cancer after exposure to MTZ compared with those who did not apply the drug. The main conclusion of their work was that there was no association between metronidazole and occurrence of cancer [6]. There is a lack of large cohort of people treated with MTZ to clearly define the risks that entails taking this medicine. In addition, this study focused on the effect of tumor induction under the influence of drugs, and not on the assessment of drug impact on a growing tumor. There is no data in the literature about the effect of MTZ on the growth of already existing tumors. The interaction of MTZ metabolites with DNA led to the examination of metronidazole for possible efficacy of radiation in cancer patients. To date, there is no accurate opinions about the mechanism by which MTZ exerts its genotoxic effect. However, there are several hypotheses trying to explain how the drug works genotoxic on aerobe cells. One of them is based on the reduction of the nitro group, another - on the metabolism of intestinal bacteria, while the latter relates to the involvement of oxidized metabolites as responsible for DNA damage. In our latest study based on the model of colon cancer cell line (DLD-1) we assessed the effect of MTZ on cell viability, DNA biosynthesis, as well as on apoptosis and necrosis using flow cytometry. Our results showed that DLD-1 cell viability was diminished in all experimental groups in comparison with the control. Moreover, we did not find any significant differences in [3H]-thymidine incorporation in various MTZ concentrations and times of observation. The reduction of cell viability was consistent with the apoptotic test [30].

CONCLUSION

In conclusion, the study showed various concentrations of MTZ in tumors according to the anatomo-pathological parameters, which is: increased MTZ concentration in tumors located in the rectum, in patients over 60, in patients without metastases to regional lymph nodes (N0) and decreasing drug concentration with increasing tumor size. Women accumulated greater amounts of MTZ in comparison to men, which testifies that its concentration in the tumor and normal colon tissue is sex-dependent. Comparison of the concentration of the drug and its metabolite in tumor and normal colon tissue shows its different reaction to MTZ.

Conflict of interest

The authors declared no conflict of interest

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