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5-Fluorouracil-metronidazole combination therapy in metastatic colorectal cancer

Clinical, pharmacokinetic and in vitro cytotoxicity studies

Zaim Bardakji[1], Jacques Jolivet[1], Yves Langelier[1], Jean-Guy Besner[2], and Joseph Ayoub[1]

[1] Institut du Cancer de Montréal, Hôpital Notre-Dame, Montréal (Québec), Canada
[2] Faculté de Pharmacie, Université de Montréal, Montréal (Québec), Canada

Summary. We have investigated the role of metronidazole (MND) combined with 5-fluorouracil (5-FU) in the treatment of metastatic colorectal cancer. MND (750 mg/m^2) was administered i.v. 1 h before 5-FU (600 mg/m^2) i.v., daily for 5 consecutive days. Treatment was repeated every 4 weeks until disease progression or prohibitive toxicity occurred. Of the 27 patients entered in the study, 4 (15%) had an objective complete or partial response lasting an average of 7 months. 5-FU toxicity was greatly enhanced by the administration of MND, however, 74% of patients having granulocytopenia ($<1500/\mu l$). We investigated the possible mechanisms underlying this enhanced 5-FU toxicity by examining whether MND modified 5-FU pharmacokinetics or whether the two drugs had a synergistic effect in vitro against the HCT-8 colon cancer cell line. While the in vitro studies failed to reveal any synergism between 5-FU and MND, pharmacokinetic evaluation revealed that 5-FU clearance was significantly reduced (26.9%, $P<0.001$) by prior MND administration. MND reduces 5-FU's therapeutic index in the treatment of colorectal cancer by impairing its clearance, which leads to increased toxicity without enhanced therapeutic efficacy.

Introduction

Colorectal cancer is the second leading cause of death among cancer patients [11]. In advanced metastatic disease, chemotherapy yields disappointing results. Only 15%–20% of patients have responses to 5-Fluorouracil (5-FU) which is considered the most active drug [6,8] and these are short-lived. One of the possible reasons underlying the failure of chemotherapy in the treatment of metastatic solid tumors is the presence of poorly vascularized hypoxic cells in the center of these tumors [3, 10]. In recent years, many investigators have shown that the nitroimidazoles, metronidazole (MND) and misonidazole can increase the cytotoxic effects of certain antitumor agents against hypoxic tumor cells [9, 12, 15, 16]. Furthemore, simultaneous administration of 5-FU and misonidazole enhanced the antitumor response in mice bearing Lewis lung carcinoma [14]. In view of the poor activity of 5-FU in metastatic colorectal cancer and the ready availability of MND, we sought to determine whether the addition of MND to 5-FU would increase its therapeutic efficacy. Since increased toxicity was noted in the first patients treated with this combination, we investigated the mechanisms behind this observation by studying the effects of MND on 5-FU pharmacokinetics and in vitro cytotoxicity.

Offprint requests to: Joseph Ayoub, Institut du Cancer de Montréal, 1560, Sherbrooke, est, Montréal, Québec, H2L 4M1, Canada

Material and methods

Chemicals

MND was supplied by Rhône-Poulenc Pharmaceutical Company (Montreal, PQ) and furosemide by Hoechst Canada Ltd. (Montreal, PQ). 5-FU and 5-bromouracil HPLC standards were obtained from Sigma Chemical Corp. (St. Louis, Mo). All chemicals were of reagent grade and purchased from the Fisher Scientific Co. (Pittsburg, Pa).

Clinical study. Patients with an histopathologic diagnosis of metastatic colorectal cancer were studied. Eligibility criteria included: age less than 75 years, a Karnofsky performance status greater than 60%, and normal liver, renal, and hematological status. Patients were treated daily with MND 750 mg/m^2, given i.v. 1 h before the administration of 5-FU 600 mg/m^2 i.v., for 5 consecutive days. This regimen was repeated every 4 weeks until disease progression or prohibitive toxicity occurred. Toxicity was assessed according to the ECOG (Eastern Cooperative Oncology Group) toxicity criteria as described in Table 1. After three cycles of treatment, responses were evaluated either by measurements of liver span, ultrasound of liver or abdominal tumor measurements, or by radiological evaluation of lung or bone metastases. Liver span was measured from the costal margin to the liver edge at three points, including the right and left midclavicular lines and the midsternal line. A response was defined as a minimum decrease of 30% in the sum of the three measurements lasting at least a month, with the absence of progressive disease at other sites. The standard criteria for objective responses were used for ultrasound and radiological measurements: *complete response:* complete disappearance of all objective evidence of disease; *partial response*: $\geqslant 50\%$ reduction in the size of all measurable lesions; *stable disease:* $<50\%$ reduction in tumor size or stabilization of the size of the lesions or 25% increase in tumor size; *progressive disease:* $<25\%$ increase in tumor size.

Pharmacokinetic study

a) 5-FU assay. 5-FU levels were measured by a modification of previously described HPLC assays [1, 2]. 5-Bromouracil was used as an internal standard. Plasma (1 ml) was deproteinated by saturation with ammonium sulfate, and 5-FU was extracted twice with 3 ml methyl ethyl ketone. The organic phase was evaporated under N_2, reconstituted in the HPLC mobile phase, and injected through an Altex 210 injector (Beckman instruments) to an octyl C8, 5-μm column (Chromatographic Sciences Company, Montreal, PQ). The mobile phase (98% phosphate buffer 0.05 *M*, pH 4.0: 2% acetonitrile) was run at 1.5 ml/min by two Beckman model 110 A pumps. The absorbance was monitored at 266 nm using a Hitachi model 100-40 spectrophotometer. Peak surface areas were measured by Perkin-Elmer (Sigma 1) integrator. Calibration curves were generated using standard solutions of 5-FU and its internal standard in blank serum. All statistical analyses for 5-FU data were done using Student's paired *t*-test.

b) MND assay. MND levels were determined by HPLC. Furosemide was used as an internal standard. MND was directly extracted from 1 ml plasma with 2 ml 50% acetonitrile in methanol, and the supernatant was directly injected on the C8 column previously used for 5-FU analysis. The mobile phase (70% acetic acid, 0.1 *M*: 30% acetonitrile) was run at 2 ml/min and the absorbance was monitored at 328 nm. Calibration curves were generated using standard solutions of MND and its internal standard in blank serum.

In vitro cytotoxicity studies

The cytotoxicity studies were done using a human colon carcinoma cell line (HCT-8) obtained from Dr A. Fuchs (McGill Cancer Center, Montreal, PQ). These cells grow continuously in monolayer with a doubling time of 18 h [18]. Cultures were maintained at 37 °C in RPMI medium 1640 supplemented with 10% fetal calf serum in 5% CO_2: 95% air. For hypoxic incubations, cells were seeded in 75-cm^2 plastic bottles. Metronidazole was added to the culture medium 24 h later and the bottles, maintained at 37 °C by incubation in a water bath, were dearated by flowing nitrogen plus 5% CO_2 (less than 10 ppm O_2) over the medium for 2 h, as described by Stratford et al. [15]. Plating efficiency was used to determine cellular viability in the presence or absence of the drugs. The colonies were scored after 10 days of incubation at 37 °C.

Results

Clinical study

1. Clinical response

Twenty-seven patients (11 male and 16 female) were entered in the study. The mean age of these patients was 60.3 (40–75) years. Almost all the patients (25/27) had liver metastases. There were six patients with lymph node involvement, three with pulmonary metastases, two with bone metastases and two with local recurrences. No patient had received previous chemotherapy. Nine of the patients (33%) were not able to complete three cycles of chemotherapy: five patients because of rapidly progressive disaease and four patients because of severe toxicity that necessitated discontinuation of the treatment. Four patients (15%) had objective evidence of remission. One patient with liver metastases has been in complete remission for at least 12 months. The other three patients had partial remission for an average of 5.3 months. Nine patients (33%) showed stabilization of the disease for an average of 5.6 months. Ten patients (37%) showed objective evidence of disease progression. In six of these the progression was very rapid, occurring after one or two cycles of chemotherapy. Our response rates were thus similar to those seen with 5-FU alone [6].

Table 1. Clinical toxicity to 5-FU + MND

Toxicity	Grade 2[a]	Grade 3[a]	Grade 4[a]
1. Granulocytopenia	4 (15%)	7 (26%)	9 (33%)
2. Anemia	6 (22%)	5 (19%)	–
3. Thrombocytopenia	5 (19%)	–	–
4. Stomatitis and oral ulceration	5 (19%)	4 (15%)	–
5. Nausea and vomiting	7 (26%)	6 (22%)	
6. Peripheral neuropathy	2 (7%)		

ECOG toxicity grading:
Granulocytopenia (10^3/μl): *grade 2:* 1.0–1.5, *grade 3:* 0.5–1.0, *grade 4:* 0.5
Anemia (Hb: g/100 ml): *grade 2:* Hb 9.5, *grade 3:* requiring transfusion
Thrombocytopenia (10^3/μl): *grade 2:* 50–90
Stomatitis and ulcers: *grade 2:* ulcers, but can eat solids, *grade 3:* ulcers, can only take liquids
Nausea and vomiting: *grade 2:* controllable, *grade 3:* intractable
[a] Number of patients affected, followed by number affected as percentage of total number of patients in parentheses in each case

2. Toxicity

Four patients developed severe nausea and vomiting along with mucositis and oral ulceration, necessitating cessation of treatment. Two patients had pancytopenia with agranulocytosis and septicemia, with one drug-related death. The details of patient toxicity according to ECOG criteria are outlined in Table 1. In total, 20/27 (74%) patients developed granulocytopenia, 11/27 (41%) anemia, and 5/27 (19%) thrombocytopenia. Nine patients (34%) suffered moderate to severe stomatitis with complicating oral candidiasis. Despite standard antiemetic medication, six patients (22%) had intractable nausea and vomiting. Two patients developed MND-related peripheral neuropathy with moderate paresthesia and weakness. Thus, gastrointestinal and hematological toxicities were much more severe than are seen with 5-FU alone [6].

Pharmacokinetic studies

To determine the mechanisms underlying the observed severe toxicity of the MND-5-FU combination, we first examined if MND modified 5-FU pharmacokinetics. As can be seen in Fig. 1 A, for ten patients studied the mean 5-FU plasma concentrations were persistently lower on day 1, i.e., after 5-FU administration alone, than on day 5, i.e., following MND administration. 5-FU pharmacokinetic

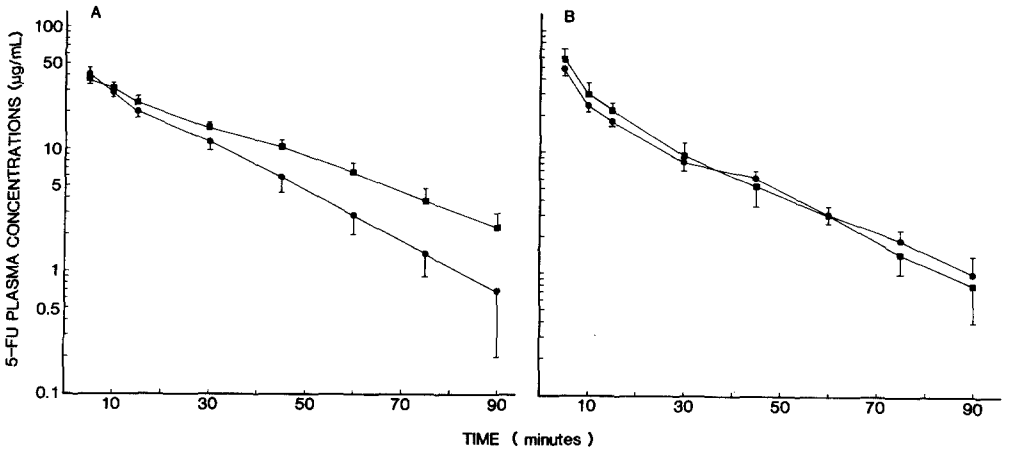

Fig. 1 A, B. 5-FU pharmacokinetics: **A** 5-FU concentrations measured in ten patients after 5-FU 600 mg/m^2 as i.v. bolus on day 1 *(circles)*, i.e., without MND pretreatment and on day 5 *(squares)*, i.e. after 4 days of 5-FU administration preceded 1 h earlier by MND 750 mg/m^2 i.v. 5-FU concentrations measured in an additional ten patients on days 1 *(circles)* and 5 *(squares)* of a 5-day 5-FU 600 mg/m^2 i.v. administration schedule. Means and SE *bars* are shown

parameters on days 1 and 5 in these ten patients are shown in Table 2. 5-FU clearance was consistently lower after MND exposure than before MND exposure. Mean 5-FU clearance dropped by 26.9% on day 5 compared with day 1 ($P<0.001$). Mean 5-FU area under the plasma concentration-time curve and elimination half-life were respectively 34% ($P<0.001$) and 38% ($P<0.005$) higher on day 5 than on day 1. To confirm conclusively that the observed decrease in 5-FU clearance on day 5 was due to MND pretreatment and not merely a consequence of the daily repetitive administration of 5-FU, we examined 5-FU pharmacokinetics on days 1 and 5 of a 5-day administration schedule in a second group of ten patients, who were not receiving MND. Mean 5-FU plasma concentrations were essentially the same on days 1 and 5 in this group (Fig. 1 B). As detailed in Table 3, the mean 5-FU clearance was also the same on days 1 and 5. Thus, MND induced a significant decrease in 5-FU clearance, which probably contributed to the increases toxicity observed. In addition, MND plasma concentrations were measured at the time of 5-FU administration (i.e., 1 h after MND administration) and were found to be 26.1 ± 5.99 µg/ml.

In vitro cytotoxicity studies

To determine whether in addition to the above pharmacokinetic interactions, other interactions at the cellular level could also play a role in the increased clinical toxicity observed, we carried out cytotoxicity studies using the human HCT-8 colon cancer cell line. In an attempt to simulate conditions observed in patients receiving 5-FU and MND, cells were exposed to 100 µg/ml MND for 1 or 24 h under aerobic conditions, followed by a 1-h incubation with 5-FU. The mean results of five experiments illus-

Table 2. 5-FU pharmacokinetic parameters in ten patients pretreated with MND

Patient	AUC (µg . min/ml)		$t^1/_2$ (min)		CL_T (l/min)		V_D (l)	
	Day 1	Day 5	Day 1	Day 5	Day 1	Day 5	Day 1	Day 5
1	1080.4	1536.0	19.2	36.3	0.694	0.488	19.23	25.56
2	421.8	560.1	8.7	18.0	1.788	1.339	22.34	34.70
3	1457.1	1677.9	19.6	24.3	0.823	0.715	23.26	25.09
4	709.3	1062.4	19.5	21.2	1.409	0.941	39.60	28.79
5	792.7	1238.2	11.1	12.9	1.261	0.807	20.15	14.48
6	1327.7	1836.5	22.0	21.9	0.753	0.544	23.91	17.18
7	942.8	1203.6	13.6	18.6	1.060	0.830	20.75	22.27
8	605.4	897.1	8.7	15.0	1.651	1.114	20.67	24.12
9	755.4	925.7	9.6	15.3	0.922	0.810	13.79	17.87
10	1033.8	1294.0	14.1	18.1	0.744	0.618	15.79	16.18
Mean ± SEM	912.6 ± 101.6	1223.2 ± 122.3	14.6 ± 1.6	20.2 ± 2.1	1.111 ± 0.126	0.821 ± 0.082	21.95 ± 2.20	22.62 ± 2.00

[a] 5-FU pharmacokinetic parameters were determined twice in ten patients receiving 5-FU 600 mg/m^2 on the first day of treatment followed in the next 4 days by the same 5-FU dose given 1 h after MND 750 mg/m^2 i.v. Pharmacokinetic parameters were determined on *day 1*, after i.v. bolus injection of 5-FU and on *day 5*, after 5-FU administration had been preceded 1 h earlier by MND. Serial blood samples were drawn on days 1 and 5 during the 3 h following 5-FU administration. The area under the 5-FU plasma concentration/time curve (AUC), 5-FU half-life ($t^1/_2$), 5-FU apparent total body clearance (CL_T) and 5-FU volume of distribution (V_D) are shown for each patient on days 1 and 5

Table 3. 5-FU pharmacokinetic parameters in ten patients not exposed to MND

Patient	AUC (μg.min/ml)		$t^1/_2$ (min)		CL_T (l/min)		V_D (l)	
	Day 1	Day 5	Day 1	Day 5	Day 1	Day 5	Day 1	Day 5
1	1524.8	1515.3	13.4	13.8	0.590	0.593	11.40	11.85
2	688.5	1025.6	13.9	17.3	1.592	1.072	31.94	26.81
3	768.8	937.9	10.0	10.2	1.279	1.049	16.70	15.38
4	774.0	753.7	10.6	12.5	1.550	1.592	23.78	28.69
5	421.2	475.4	8.1	7.5	1.780	1.570	20.92	17.18
6	881.4	747.8	21.2	20.4	1.077	1.270	32.96	37.37
7	1258.1	1062.0	23.6	26.0	0.596	0.706	20.28	26.46
8	559.0	656.6	4.5	4.0	1.340	1.140	8.75	6.64
9	2157.6	1016.2	14.6	25.0	0.417	0.886	8.80	31.97
10	1019.5	798.1	11.8	18.6	0.932	1.190	15.85	31.91
Mean ± SEM	1005.3±164.2	898.9±90.1	13.2±1.8	15.5±2.3	1.115±0.149	1.107±0.103	19.14±2.73	23.43±3.18

[a] 5-FU pharmacokinetic parameters were determined in ten patients on days 1 and 5 of a 5-day administration schedule for 5-FU (600-mg/m^2 i.v. bolus daily). Serial blood samples were drawn during the 3 h following 5-FU administration on both days. The area under the plasma 5-FU concentration/time curve (AUC), 5-FU half-life ($t^1/_2$), 5-FU apparent total body clearance (C_{LT}) and 5-FU volume of distribution (V_D) are shown for each patient on days 1 and 5

trated in Fig. 2 demonstrate that under these conditions MND does not modify the cytotoxicity of 5-FU. In another set of experiments cells were incubated for 10 days with both MND (50 μg/ml) and 5-FU. This treatment also failed to reveal any enhancement of cytotoxicity by MND (data not shown). Finally, to mimic the conditions used by Stratford et al. [15] to demonstrate the increased cytotoxicity of chemotherapeutic agents in the presence of misonidazole, we exposed HCT-8 cells, following a 2-h MND 5 mg/ml pretreatment in hypoxic conditions, to either a 1-h or a 10-day exposure to 5-FU. Here again, MND did not modify the cytotoxic effects of 5-FU. Control experiments had shown that incubation of cells in the presence of MND alone under aerobic conditions (5 mg/ml for 2 h or 100 μg/ml for 24 h or under hypoxic conditions (5 mg/ml for 2 h) had no effect on cell viability.

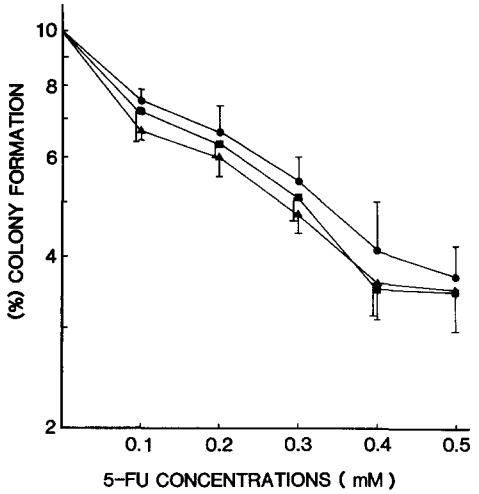

Fig. 2. Survival curves for HCT-8 cells exposed to varying concentrations of 5-FU for 1 h in air. The *circles* represent cells treated only with 5-FU; the *squares*, cells pretreated with 50 μg/ml MND 1 h before treatment with the combination of MND 50 μg/ml + 5-FU; the *triangles*, cells pretreated with 50 μg/ml MND 24 h before treatment with the combination of MND 50 μg/ml + 5-FU. Means of five experiments along with the standard error *bars* are shown

Discussion

MND in the doses and schedule used in this study increased the toxicity of 5-FU without augmenting its beneficial effects. This increased toxicity is related to a MND-induced decrease in 5-FU clearance, leading to a greater exposure of normal tissues to the drug and consequently more side effects. Unfortunately, the increased tumor exposure to 5-FU did not appreciably increase its antitumor activity, and the drug's therapeutic index was decreased. There was probably no additional cellular interaction leading to increased cytotoxicity, since MND and 5-FU studies revealed no synergistic cytotoxicity between MND and 5-FU under either standard or hypoxic conditions.

Our study did not contain a control group of patients treated with 5-FU alone. Moertel et al. [6] utilized a similar 5-FU administration schedule to the one we used (without MND) and obtained response rates (12% partial remission and 30% stable disease) similar to those observed in our patients. However, he reported only 19% ECOG grade 3 and 8% grade 4 leukopenia, as against our 26% and 33%, respectively. Furthermore, the 43% incidence of anemia, 19% of thrombocytopenia and 34% of severe stomatitis seen in our study are toxicities that are not usually encountered with 5-FU alone. To our knowledge, there is only one reported clinical trial examining 5-FU in combination with a nitroimidazole. Spooner et al. [14] studied the combination of misonidazole and 5-FU in 15 patients with advanced colorectal cancer and also found an increased incidence and severity of gastrointestinal toxicity and a slightly increased incidence of leukopenia.

The MND-induced decrease in 5-FU clearance is in agreement with the data of McDermott et al. [5], who in five patients treated with misonidazole and 5-FU observed that 5-FU clearance following the administration of 1.0 g/m^2 5-FU i.v. was significantly reduced by misonidazole

given p.o. 2 h before 5-FU therapy. Again, 5-FU clearance correlated inversely with the severity of gastrointestinal toxicity, which was greatly enhanced by the administration of misonidazole.

It is generally accepted now that nitroimidazoles enhance the cytotoxic effects of certain chemotherapeutic agents – mainly bifunctional alkylating agents and nitrosoureas – against hypoxic tumor cells in vitro [10, 13, 15, 16]. This enhancement depends upon many factors: the cell type, the chemotherapeutic agent used, the nitroimidazole's concentration, the degree of hypoxia, and the timing of administration of the nitroimidazoles and the chemotherapeutic agent relative to each other. The absence of enhanced cytotoxicity in our experimental model is probably related to the lack of a specific interaction between 5-FU and MND, since the few experimental studies [4, 17] of the combination have failed to confirm the preliminary results reported with 5-FU misonidazole [14]. One cannot rule out from this one model, however, that synergistic toxicity could not have occurred in normal tissues in vivo.

In conclusion, MND substantially increases 5-FU toxicity when administered to patients with metastatic colorectal cancer, and further use of this combination cannot be recommended.

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