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THE IN VIVO RESPONSE OF A C3H MAMMARY CARCINOMA TO TREATMENT WITH MISONIDAZOLE, CYCLOPHOSPHAMIDE AND RADIATION

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Abstract

The potential chemosensitizing effect of the nitroaromatic radiosensitizer misonidazole (MISO) on the alkylating agent cyclophosphamide (CTX), and the interactions of these agents with radiation, have been investigated in a C3H mammary carcinoma in CDF_1 mice. MISO at 1 000 mg/kg caused a small increase in tumour growth time (TGT; time to reach 3 times treatment volume) from 3.6 days to 4.5 days. CTX (100 mg/kg) increased the TGT to 15.7 days. The combined treatment of MISO and CTX given with intervals of either 15 min or 4 h increased the TGT to 23.3 and 23.8 days respectively. The radiation enhancement ratio (ER) was found to be 2.13 and 1.10 for MISO administered before or after x-rays respectively. The corresponding ERs for CTX were 1.16 and 1.22. The two drugs given in combination resulted in significant radiation ERs of 2.68 (both drugs given within 30 min before x-rays), 3.00 (MISO 30 min before and CTX $3\frac{1}{2}$ h after x-rays) and 1.40 (both drugs given after x-rays). In contrast to what has previously been reported, and in contrast to the tumour regrowth delay data, the results of the tumour control experiments were found to reflect no more than an additive action of the two drugs when used together with radiation in vivo.

Key words: Experimental tumours, misonidazole, cyclophosphamide, radiation, chemosensitization, in vivo.

Since the initial observation in 1980 by Rose et al. (1) that the nitroimidazole misonidazole (MISO) enhanced the cytotoxic action of some alkylating anti-cancer agents, several investigators have shown the chemosensitizing ability of nitroimidazoles in different animal models (see review by Siemann (2)). As a consequence of such studies, the potential therapeutic benefit in patients is currently being investigated by phase II clinical trials in the treatment of malignant melanoma and renal cell carcinoma (3, 4).

Several mechanisms have been proposed for the chemopotentiation observed in vivo. These include sensitizer-induced changes in pharmacokinetics, preferential killing of hypoxic cells or a possible interference with the repair of potentially lethal damage (2, 5, 6). However, despite major investigational efforts, the exact mechanisms behind chemosensitization still remain unsettled.

We have investigated the interaction between MISO, the alkylating agent cyclophosphamide (CTX) and radiation in a C3H mouse mammary carcinoma using two in situ assays. The purpose of the present study was to evaluate drug–drug and drug–radiation interactions, as a function of dosage and time interval between the involved agents.

Material and Methods

Animal tumour system. The C3H/Tif mammary carcinoma was grown in the right rear foot of 10–12-week-old male C3D2F1/Bom mice. The derivation and maintenance of this tumour has been described previously (7). Non-anaesthetized mice were treated when tumours were on average 200 mm^3 (range 150–257 mm^3), determined by the formula: $\pi/6 \times D_1 \times D_2 \times D_3$, where the Ds represent the three orthogonal diameters.

Irradiation. Irradiation was given as 250 kV x-rays (10 mA, HVL 3.1 mm Cu, dose rate 2.26 Gy/min). The mice were placed in a lucite jig with the tumour-bearing leg exposed, loosely taped to the jig and immersed in a water bath to ensure equal dose distribution in the tumour.

Drugs. MISO was dissolved in sterile isotonic saline at

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room temperature and administered intraperitoneally (i.p.) in a volume of 0.04 ml/g of body weight. CTX was dissolved in sterile distilled water and injected i.p. at a volume of 0.02 ml/g of body weight.

Tumour growth delay. The tumour volume was measured on a daily basis and the response evaluated in terms of tumour growth time (TGT), defined as the time required for a tumour to reach three times the treatment volume. The exponential regrowth phase was used to calculate the volume doubling time (DT). All calculations were based on individual growth data. Each treatment included 6–20 mice. To eliminate the possible biasing effect of treatment-induced alterations in the DT, a corrected TGT was calculated for each animal. This was done by multiplying the observed TGT by the ratio between the known DT for controls and the observed DT for the treated animal.

Local tumour control. The effect of graded doses of radiation alone or in combination with drugs was evaluated as the radiation dose required to produce local tumour control in 50% of the treated animals (TCD_{50}). Tumour control was defined as complete absence of macroscopic relapse within 90 days. A microscopic diagnosis was carried out on those mice in which there was some doubt about tumour control. In these cases only evidence of viable tumour cells (e.g. multiple mitoses) caused the tumour to be classified as a treatment failure. Less than 1% of the mice died without tumour before 90 days and were excluded from the study. All experiments included 6–12 mice per dose point and were repeated at least once. Dose response curves were based on 5–18 dose points per treatment. Data were analyzed using a logit analysis (8).

Enhancement ratios. The modifying effect of drug dose on radiation-induced tumour control was evaluated by two different methods. The isodose drug enhancement ratio was calculated as the ratio between the observed response at a given dose of CTX with or without MISO. The isoeffect drug enhancement ratio was determined as the dose of CTX required to achieve a certain level of tumour control with or without MISO.

The effect of a fixed drug dose on the radiation response was expressed as a radiation enhancement ratio (ER). It was calculated as the TCD_{50} for radiation alone relative to the corresponding value for radiation combined with drugs:

$$ER = \frac{TCD_{50}\ (\text{radiation})}{TCD_{50}\ (\text{radiation} + \text{drug})}$$

Results

Tumour regrowth delay assay. Table 1 lists the DT and TGT after treatment with MISO (1 000 mg/kg) and CTX (100 mg/kg) alone or in combination with either a 15-min or a 4-h interval. A large single dose of MISO (1 000 mg/kg) resulted in a small but significant increase in

Table 1

Volume doubling time and tumour growth time after treatment with misonidazole (MISO) and cyclophosphamide (CTX) alone or in combination at varied time intervals

		Volume doubling time		Days to reach 3 times treatment volume (TGT)			
Treatment	n	(days)	t-test	Observed TGT	t-test	Corrected TGT[a]	t-test
Untreated control	173	2.6 (2.5–2.7)		3.6 (3.5–3.7)			
MISO	23	2.9 (2.6–3.2)	NS[b]	4.5 (4.1–4.8)	$p < 0.001$[b]	4.3 (3.8–4.7)	$p < 0.01$[b]
CTX	42	2.9 (2.6–3.2)	NS[b]	15.7 (14.1–17.3)	$p < 0.001$[b]	15.2 (13.4–17.0)	$p < 0.001$[b]
MISO – 15 min – CTX	25	3.1 (2.9–3.4)	$p < 0.001$[b]	23.3 (20.9–25.8)	$p < 0.001$[b]	19.7 (17.6–21.9)	$0.01 > p > 0.001$[c]
MISO – 4 h – CTX	16	3.3 (3.0–3.7)	$p < 0.001$[b]	23.8 (21.2–26.3)	$p < 0.001$[c]	19.4 (16.7–22.0)	$0.02 > p > 0.01$[c]
MISO: 400 – 2 h – 300 – 2 h – 300 mg/kg – 15 min – CTX	6	3.3 (2.8–3.8)	$p < 0.05$[b]	21.8 (18.9–24.6)	$p < 0.001$[c]	17.9 (14.5–21.4)	NS[c]

MISO 1 000 mg/kg; CTX 100 mg/kg. Numbers in parentheses are 95% confidence interval on mean. All calculations were based on individual growth curves.

[a] Corrected tumour growth time = $TGT_{obs} \times (DT_c/DT_{obs})$. See text.

[b] Student's t-test; treated animals versus untreated controls.

[c] Student's t-test; combined treatment versus CTX (100 mg/kg).

tumour regrowth from 3.6 days in untreated animals to 4.5 days ($p < 0.001$). CTX caused a marked increase in TGT from 3.6 to 15.7 days. When the drugs were given together an additional increase in regrowth delay was observed, the TGT values being 23.3 days (15-min interval) and 23.8 days (4-h interval). The effect of prolonged exposure to MISO prior to CTX administration was also investigated. This was done by giving MISO at a total of 1 000 mg/kg divided into 3 fractions within 4 h prior to CTX. However, the tumour response (TGT 21.8 days) was not different from that seen after a single MISO injection prior to CTX treatment.

The volume doubling time (DT) was significantly influenced by the combination treatment. In untreated control tumours the DT was 2.6 days. This value, which was based on data from 30 individual experiments including a total of 173 mice, was very constant and showed no significant intra- or interexperimental variation. Neither MISO nor CTX caused any significant change in DT when the drugs were administered as single agents (5% significance level). However, when the two drugs were combined a significant increase in DT was observed ($p < 0.001$). Eventually this DT effect could lead to an overestimation of the combined drug cytotoxicity, since the observed increase in TGT might not be entirely due to a decrease in cell survival (9). To overcome this problem we estimated a TGT which was corrected for the individual variations in DT. The result of this correction is listed in Table 1 (right). As is seen, the additional delay caused by the combined drug treatment, when compared to CTX alone, was reduced from 8 days to approximately 4 days (single MISO injections prior to CTX). These corrected values were still significantly different from that of CTX alone. On the other hand, the enhancement in tumour response after multiple small MISO injections prior to CTX became non-significant after correction. The discrete action of MISO alone was taken into account by calculating a theoretical (expected) TGT, based on additive drug cytotoxicity. Assuming independent action, the additive drug effect was estimated to be equivalent to a TGT of 16.1 days (15.2 days from CTX + 0.9 day from MISO). A comparison with the observed TGTs showed that both at the 15-min and the 4-h interval the combined effects were greater than that expected on an additive basis ($p < 0.02$ and $p < 0.05$ respectively).

Effect of drug dose on tumour control. The ability of drugs to modify the radiation-induced tumour control was investigated by giving varied drug doses 4 h after a fixed radiation dose of 45 Gy. Radiation was given in order to obtain tumour control, which could not be achieved with the drugs alone. The drugs were administered after x-rays in order to avoid any direct radiosensitization. The observed dose response curves are shown in Fig. 1. In the left figure is shown the tumour control obtained when graded doses of MISO were given in a regimen consisting of:

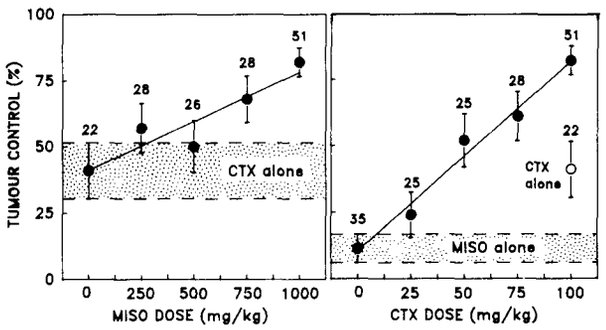

Fig. 1. The relationship between injected drug dose and modification of tumour control. All mice received 45 Gy 4 h before drug treatment with either graded MISO doses plus a constant dose of CTX (100 mg/kg; left curve) 15 min later, or graded CTX doses 15 min after a constant dose of MISO (1 000 mg/kg; right curve). The number of mice per dose point is indicated. Error bars represent ± 1 SD.

45 Gy – 4 h – MISO – 15 min – CTX (100 mg/kg). The right panel shows the corresponding line for graded doses of CTX given in the same schedule with MISO at a constant dose of 1 000 mg/kg. A linear correlation between drug dose and tumour control was found with both drugs, the relationship being most evident for the CTX dose—response curve. Only maximally tolerated doses of both MISO (1 000 mg/kg) and CTX (100 mg/kg) resulted in a significantly improved local control when compared to that of 45 Gy + CTX alone. The isodose drug enhancement ratio at this dose was 2.0, calculated as the ratio between the response for the combined drug treatment (82%) and CTX alone (41%). From Fig. 1 (right panel), the isoeffect drug enhancement ratio for MISO at 1 000 mg/kg, was found to be 2.4, calculated as the CTX dose required to control 41% of tumours without MISO relative to the CTX dose required to obtain the same control with MISO (100 mg/kg relative to 42 mg/kg = 2.4).

Drug sequence and time interval. The influence of varying the sequence and time interval between MISO and CTX was tested using both a regrowth delay and a tumour control assay. As previously stated, the data from the regrowth delay experiments (Table 1) showed no significant alteration in tumour response when MISO was administered either 4 h or 15 min prior to CTX. Nor did it influence the tumour regrowth when MISO was administered in small fractions within the same time interval.

The influence of drug sequence and timing was more intensively studied in a tumour control assay. The results of these experiments are listed in Table 2. To obtain a graded response, all tumours received 39 Gy plus adjuvant treatment. CTX was administered 4–4¼ h after x-rays. The time of MISO administration was then varied from 15 min to 8 h after radiation, i.e. from ¾ h before to 4 h after CTX administration. Radiation alone (39 Gy) did not produce any tumour control, whereas 39 Gy + CTX resulted in 22% of the tumours

Table 2

The influence of time interval between administration of misonidazole (MISO) and cyclophosphamide (CTX) on local tumour control. All tumours received radiation (39 Gy) 4 h before administration of CTX

Adjuvant treatment	Tumour control (%)	Fisher's exact test*
39 Gy	0/9 (0)	
39 Gy – 4 h – CTX	4/18 (22)	
39 Gy – 15 min – MISO – 3 h 45 min – CTX	8/12 (67)	p = 0.02
39 Gy – 4 h – MISO – 15 min – CTX	12/19 (63)	p = 0.02
39 Gy – 4 h – CTX – 15 min – MISO	8/11 (73)	p = 0.02
39 Gy – 4 h – CTX – 4 h – MISO	6/12 (50)	NS

CTX 100 mg/kg; MISO 1 000 mg/kg
* Compared to response obtained by: 39 Gy-4 h-CTX (22%). 5% significance level.

being controlled. When MISO was added the response rates were 50–73%. The tumour control was not significantly changed within the used time intervals although a decreasing tendency was observed when MISO was given 4 h after CTX. At this time interval the response of the combined treatment was not significantly different from that of 39 Gy plus CTX alone.

TCD_{50} assay. The interactions between drugs and graded doses of radiation was studied using a local tumour control (TCD_{50}) assay. The results are presented in Table 3 and Fig. 2 (curve number in parentheses). The hypoxic radiosensitizing effect of MISO was expressed by a significant reduction of the TCD_{50} when the drug was administered 30 min before radiation (c), corresponding to an ER of 2.13 ($p < 0.001$). When MISO was given 4 h after x-rays (b) the ER was found to be 1.10 ($p < 0.001$). CTX also gave rise to a significantly enhanced radiation response, regardless of whether the radiation was given 15 min after (d) or 4 h before (e). The radiation ERs were 1.16 and 1.22 respectively ($p < 0.001$ at both intervals). When MISO and CTX were administered in combination before x-rays (f) the observed reduction in TCD_{50} corresponded to an ER of 2.68 ($p < 0.001$). The greatest radiation enhancement in the study was observed with MISO given before and CTX after x-rays (g; ER = 3.00; $p < 0.001$). But when MISO was administered after x-rays (h), a distinct decrease in radiation enhancement was observed, due to the loss of hypoxic radiosensitization by MISO itself, the ER being reduced from 3.00 to 1.40 ($p < 0.001$). To determine whether the observed effect of combined drug therapy on radiation response reflected more than the additive effects of the single drugs, an expected (additive) ER was calculated as the product of the observed ERs from the two single drug treatments. The observed ER for combined drug treatments were then compared to this expected value and a potential supraadditive effect was tested for by statistical comparison of these values. The expected ERs for each combined drug treatment are listed in Table 3. As is seen from this table, none of the observed ERs was significantly different from what would be expected on an additive basis.

Table 3

The effect on local tumor control (TCD50) and enhancement ratio (ER) of combinations of radiation, CTX (100 mg/kg) and MISO (1 000 mg/kg)

Treatment	No. of mice	TCD_{50} Gy	Observed ER single drug	Observed ER two drugs	Expected ER[a] two drugs	t-test[b]
Radiation alone (a)	542	54.42 (53.46–55.40)	—	—	—	
MISO – 30 min – RAD (c)	168	25.51 (24.79–26.25)	2.13 (2.07–2.20)			
CTX – 15 min – RAD (d)	112	46.76 (44.54–49.09)	1.16 (1.12–1.21)			
MISO – 15 min – CTX – 15 min – RAD (f)	50	20.29 (18.36–22.44)		2.68 (2.48–2.90)	2.47 (2.39–2.55)	NS
MISO – 30 min – RAD (c)	168	25.51 (24.79–26.25)	2.13 (2.07–2.20)			
RAD – 4 h – CTX (e)	87	44.47 (41.98–47.11)	1.22 (1.17–1.28)			
MISO – 30 min – RAD – 3.5 h – CTX (g)	62	18.14 (16.94–19.43)		3.00 (2.83–3.18)	2.60 (2.51–2.69)	NS
RAD – 4 h – MISTO (b)	168	49.51 (48.07–50.98)	1.10 (1.07–1.13)			
RAD – 4 h – CTX (e)	87	44.47 (41.98–47.11)	1.22 (1.17–1.28)			
RAD – 4 h – MISTO – 15 min – CTX (h)	127	38.98 (36.72–41.38)		1.40 (1.33–1.47)	1.34 (1.28–1.40)	NS

Numbers in parentheses represent 95% confidence limits. Letters in parentheses indicate label in Fig. 2.
[a] Expected ER is the product of the ER for single treatments.
[b] 5% significance level.

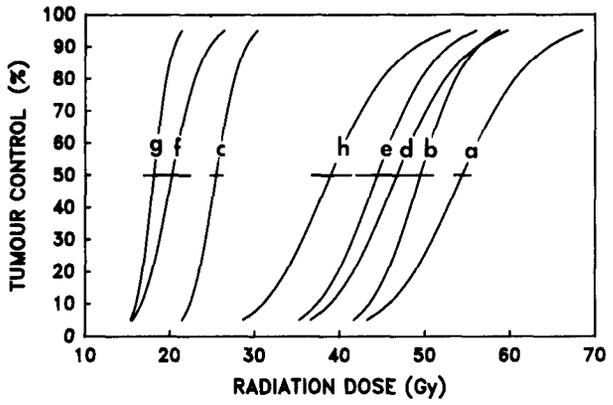

Fig. 2. TCD_{50} curves showing the radiation response of a C3H mammary tumour. (a) radiation along; (b) radiation-4 h-MISO; (c) MISO-30 min-radiation; (d) CTX-15 min-radiation; (e) radiation-4 h-CTX; (f) MISO-15 min-CTX-15 min-radiation; (g) MISO-30 min-radiation-3.5 h-CTX; (h) radiation-4 h-MISO-15 min-CTX. Error bars represent 95% confidence limits.

Discussion

The aim of the present investigation was to evaluate the dose–response relationship and the effect of the nitroaromatic drug MISO used in combination with the alkylating agent CTX, and the interaction of these agents with radiation. The tumour response was evaluated by two different in situ assays: the tumour regrowth delay assay and the local tumour control assay.

A linear correlation for the modifying effect of drug doses on radiation-induced tumour control was found (Fig. 1). This was true for both drugs at doses up to 100 mg/kg (CTX) and 1 000 mg/kg (MISO). The dose–response relationship for these drugs is in agreement with reports using the regrowth delay endpoint without irradiation (10, 11). However, other investigators using cell survival and tumour regrowth delay assays (12, 13) found a plateau for CTX doses over 50 mg/kg, and in one case a decrease in enhancement was reported (12). For MISO a plateau at a dose of 250 mg/kg in the TGT assay and 500 mg/kg in the cell survival assay has been observed (12, 14). Based on the dose–response curves in our study the drug ERs were found to be about 2. Other investigators have found similar enhancement in other animal strains and tumour systems. In KHT tumours, using growth delay assay, the chemosensitization by MISO on CCNU (20 mg/kg) gave ERs from 1.3 (MISO 250 mg/kg) to 1.9 (MISO 1 000 mg/kg) (15). Similar drug doses used in an excision assay produced dose-modifying factors of 1.9 and 2.4 (16).

The influence of sequence and time interval between the hypoxic cell sensitizer and the chemotherapeutic agent has been reported from several other investigators. Generally, a maximum effect is seen when MISO is given 0.5–2 h before CTX, with a subsequent loss of interaction at larger intervals (10–12). In the present study we did not manage to demonstrate any significant importance of sequence and time interval between the two drug treatments, although there was a slight drop in enhancement when MISO was given 4 h after CTX, as compared to simultaneous treatment (Tables 1 and 2). Thus, in vivo there seems to be a fairly broad time span in which the possible sensitizing interaction may occur.

When MISO and CTX at maximal doses were combined with x-rays a significant improvement in radiation response (TCD_{50}) was observed for all treatment sequences and combinations when compared to radiation alone. However, the observed enhancement was in all cases found to be that expected on a purely additive basis. The few other reports on this topic show some discrepancy. The present findings are in close accordance with a previous report from our laboratory (17), on the interactions of nimorazole (a MISO analogue) with CTX and radiation. In contrast, other studies have shown supraadditive cell killing and increased regrowth delay when MISO was combined with CCNU and radiation in KHT sarcomas (18, 19).

In the regrowth delay experiments the combined drug treatment caused a significant increase in DT and TGT. The effect on DT may reflect a selection for slowly proliferating cells, or it may reflect a 'tumour bed effect' similar to that observed after x-rays. However, even after correction for the individual changes in DT, the increase in TGT was still significantly greater than that expected on an additive basis. In contrast to the results from the TCD_{50} experiments, these results suggest that MISO is capable of sensitizing tumour cells to the cytotoxic action of CTX, resulting in supraadditive cell killing.

The observed discrepancy between the conclusions drawn from the regrowth delay and the tumour control assays may reflect differences in the treatment response of different tumour subpopulations. In a recent study we have investigated the effect of combined MISO and CTX on aerobic and hypoxic tumour cells in vivo (20). It was found that the effect on aerobic cells was more than additive, suggesting a chemosensitizing effect on these cells. No synergistic effect was found on radiobiologically hypoxic cells. These results are consistent with the present observations. As it is believed that aerobic cells dominate the growth of tumours, the sensitization and killing of these cells would be expected to result in a significant growth reduction. On the other hand, the control of a tumour is dependent on the killing of radioresistant hypoxic cells. No sensitization of these cells was found (20), which is in agreement with the lack of any significant supraadditive radiation enhancement in the TCD_{50} data presented here. The present results thus emphasize the importance of choosing endpoints, which are biologically and/or clinically relevant—in order to avoid erroneous conclusions to be drawn.

In conclusion, this paper has presented in situ data on

the chemosensitizing effect of MISO on the response of a solid tumour to CTX, and the interactions of these agents with radiation. A linear relationship was found for the modification of tumour control as a function of drug doses when evaluated in combination with a fixed radiation dose. The combined treatment caused a significant increase in tumour growth delay. When used together with x-rays, different drug radiation schedules revealed no more than an additive effect on tumour control by the two drugs.

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