Neutral Metoclopramide Induces Tumor Cytotoxicity and Sensitizes Ionizing Radiation of a Human Lung Adenocarcinoma and Virus Induced Sarcoma in Mice

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Radiation induced cytotoxicity was potentiated by neutralized metoclopramide (nMCA; Neu-Sensamide™, Oxigene Inc) when a human lung adenocarcinoma (H2981) transplanted into scid mice and an adeno-type 12 virus induced mouse sarcoma (A12B3) inoculated into CBA mice were exposed in vivo to low dose radiation at single doses of 1 and 2 Gy respectively. However, when the radiation dose was increased to 6, 10 or 18 Gy (single dose) and combined with a single dose nMCA (2 mg/kg), tumor cytotoxicity was not sensitized by the combination treatment. A fractionated dose of ionizing radiation (3 × 1 Gy) in combination with nMCA at a repeated dose of 3 × 10 mg/kg body weight (1 dose/day, i.m.) significantly increased cytotoxicity in H2981 compared with radiation given alone. nMCA alone also had a statistically significant dose dependent cytotoxic effect on H2981 growth when it was administered as repeated doses (8 doses) at 2 mg/kg or 10 mg/kg (1 dose every second day), and a similar result was achieved at 20 mg/kg but not at 2 and 10 mg/kg in the A12B3 tumor. In addition, the tumor volume at the start of treatment was important for the anti-tumor effect of nMCA (i.e. the larger initial tumor volume gave less effect on tumor growth). Taken together, our data propose that the mode of action of nMCA is different from radiation, and hence the two mechanisms are at least additive when in combination with lower radiation doses. The data further suggest that the cytotoxic mechanism is consistent with potentiating apoptosis because low and repeated doses of radiation (1–2 Gy), which are known to increase cytotoxicity by apoptosis, are sensitized by nMCA but not high doses and nMCA has more potent anti-tumor effects against H2981 tumors which have a higher constitutive apoptotic fraction of cells than A12B3.

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The N-substituted carboxamide derivative metoclopramide (MCA) has been available in the clinic for more than 30 years as an antiemetic (1). However, in the last five years the interest in MCA has been focused on its sensitizing properties of radio- and chemotherapy both in animals (2–6) and in the clinic (7, 8). Three potential modes of action for MCA have been identified: a) MCA can induce DNA damage and inhibit DNA repair (9, 10); b) MCA can in the presence of radiation react with cellular reducing components such as glutathione and may thereby increase radiation induced DNA damage (8) and c) MCA induces apoptosis in human leukemic HL-60 cells (11).

Radiation induced cytotoxicity is generally believed to occur via induction of DNA damage causing clonogenic cell killing (12). Thus, the cytotoxicity is caused by massive DNA damage, which is not fully repaired by the cell, and as such the cells ability to replicate is inhibited and cytotoxicity by mitotic cell death or necrosis is induced (13). Clinically radiation is normally given in a fractionated dose schedule at low doses of 1–2 Gy per fraction (14), and during recent years it has been shown that relatively low or moderate doses of ionizing radiation (15–17) and several classes of cytotoxic drugs, such as etoposide, cisplatin and DNA alkylating agents (18, 19) can preferentially induce cell death by apoptosis. Cell death by apoptosis involves metabolic changes and altered gene expression which results in cell shrinkage, membrane blebbing, nuclear condensation and increased activity of endonucleases causing DNA degradation (20, 21). Hence, because MCA has been shown to sensitize radiation at relatively low radiation doses (2) and induces apoptosis in vitro in HL-60 cells (11), apoptosis may be another possible mechanism to explain the radiosensitizing properities of MCA as an enhancer of cytotoxicity in vivo.

A new formulation of MCA (Neu-Sensamide™, Oxigene Inc.), conformationally altered by neutralization of pH

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and with a reduced side effect profile (8, 11), potentiates cytotoxicity induced by ionizing radiation at relatively low doses in the human lung adenocarcinoma (H2981) transplanted into scid mice (2). Here we further characterize the anti-tumor properties of neutralized metoclopramide (nMCA) using two different tumor models, by varying the doses of nMCA and ionizing radiation, or by modifying the dose schedules of nMCA and radiation given alone or in combination. A preliminary report of these data was given at the 15th annual meeting of ESTRO (22).

MATERIAL AND METHODS

Mice. Six to eight-week-old scid mice (23) and 10–15-week-old CBA mice of both sexes were used. The average body weights for scid mice were 20 ± 3 g for female and 25 ± 3 g for male mice, and for CBA mice the body weights were 21 ± 2 g and 29 ± 4 g respectively. The animals were maintained under sterile but not specifically pathogen-free conditions and were treated according to the Swedish guidelines for humane treatment of laboratory animals and the experiments were approved by the ethical committee for animal research in Malmö/Lund, Sweden.

Tumor lines. The tumor line H2981 originated from a human lung adenocarcinoma which has been established in in vitro cell culture (24) and implanted into scid mice. An immunogenic (adeno type 12 virus induced) mouse sarcoma tumor line A12B3 was carried in CBA mice (10). The tumor grafts were serially transplanted by subcutaneous inoculation of tumor cell suspension into the right flank of the mice. The animals were randomly divided into treatment groups, where no significant difference of sex, body weight or initial tumor volume between the groups was observed.

Drug treatments. nMCA [4-amino-N(2-diethylaminoethyl)-5-chloro-2-methoxy-benzamide monohydrochloride] (Neu-Sensamide™, Oxigene, Europe AB, Lund, Sweden) was provided as a 100 mg/ml (calculated as free base) sterile injectable concentration, pH 6.5–7.0. The formulation was diluted with sterile saline and was sterilized again by filtration (0.22 μm) at concentrations of 0.5 and 2.5 mg/ml and 100 μl of the drug was injected intramuscularly (i.m.) into scid mice and intraperitoneally (i.p.) into CBA mice 1 h before irradiation at final doses of 2, 10 or 20 mg/kg body weight. Control animals were injected with an equal volume of sterile saline as a placebo control.

Irradiation. Radiation was performed with an x-ray source (50 kV, 4.76 Gy/min). The radiation treatment was given to the animals 1 h after injection of nMCA. The tumors were locally irradiated with a single dose (1–18 Gy) or a fractionated dose of 3×1 Gy (1 Gy/day) while the mice were under anaesthesia (Sombrevin®).

Tumor volume measurements. The size of the tumors were measured over a period of 8 to 24 days for the H2981 in scid mice and 7 to 17 days for the A12B3 in CBA mice after inoculation of the tumors. The animals were sacrificed when the tumors had reached a maximal perpendicular axis measurement of 15×15 mm. The tumor volumes were calculated as volume $= L \times W^2 \times 0.4$ where L is the length (mm) and W (mm) is the width of the tumor (25). At the first day of radiation (7–10 days after inoculation) average tumor volume for H2981 was 63 ± 27 mm^3. In the anti-tumor experiments the H2981 tumor volume varied between 28 and 141 mm^3 with an average volume of 70 ± 30 mm^3, $n = 155$. The tumor volume for the A12B3 mouse sarcoma varied between 11 and 124 mm^3 with an average of 51 ± 27 mm^3, $n = 92$.

Evaluation of tumor response. Relative tumor size (RTS) was calculated as tumor volume at time of measurement divided by the tumor volume at the first day of treatment. To get normal distribution values log RTS was used and log RTS versus time was plotted. Based on the RTS data the area under the curve (AUC), tumor doubling time (TDt) and specific growth delay (SGD) were calculated and used as indicators of treatment efficacy. The growth curves were fitted by polynomial regression and used to calculate the time taken for each tumor to grow to its double size. SGD was calculated according to Berman & Steel (26) as SGD = (TDt-TDc)/TDc, where TDt equals tumor doubling time and TDc equals mean tumor doubling time for the control group.

Scoring of apoptosis. Animals were killed and the excised tumors fixed in neutral buffered formalin and embedded in paraffin blocks from which 4–5 μm sections were prepared and stained with heamatoxylin and eosin (27). The assay for scoring the presence of apoptotic cells in the tumor was performed by microscopic examination of stained tumor sections at a magnification of 400 X. A total of five fields of non-necrotic areas were selected from two slide preparations per tumor. The total number of nuclei from apoptotic, mitotic and normal cells were counted (about 150 cells per field for H2981 and 300 cells for A12B3) by three independent examiners and the average percentage of apoptotic cells was reported as the constitutive level of apoptosis in the tumor.

Toxicity. Drug toxicity was assessed as a change in body weight and by acute toxic symptoms and survival during the treatment period.

Statistics. Calculations were carried out by SPSS statistical software. For overall comparison between two groups a two-tailed Student's t-test, at a significance level of 0.05, was used. For data showing skewed distribution the Mann-Whitney U-Wilcoxon Rank Sum W test was used.

RESULTS

The effect of MCA on tumor cytotoxicity from increased doses of radiation. nMCA has been shown to sensitize the cytotoxicity induced by ionizing radiation at a single dose of 1 Gy in the human lung adenocarcinoma (H2981)

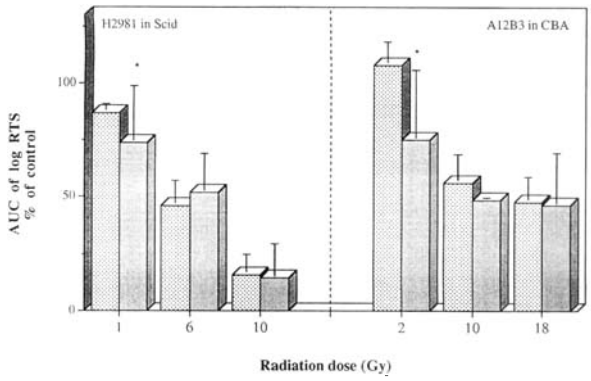

Fig. 1. The effect on AUC of log RTS, of increased radiation doses when combined with nMCA, of two different tumor lines, a human adenocarcinoma H2981 xenografted to scid mice and a mouse sarcoma A12B3 inoculated into CBA mice. The results are expressed as % of non-irradiated control tumors, where the left bar (dotted) shows irradiated tumors and the right bar (black) represent data from tumors treated with radiation in combination with a single dose of nMCA at 2 mg/kg. (*) indicates a statistically significant difference compared to ionizing radiation only, $p < 0.05$, t-test.

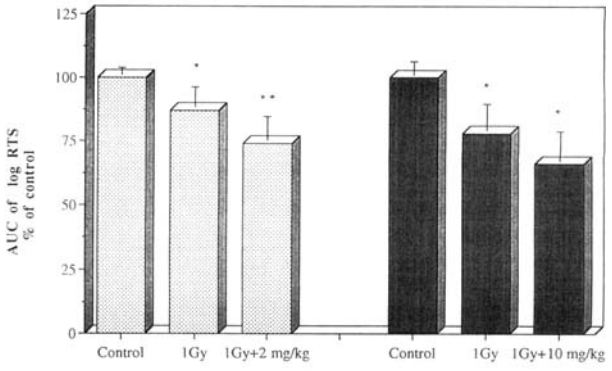

Fig. 2. Dose escalation of nMCA (neutral metoclopramide) from a single dose of 2 to 10 mg/kg did not improve the radio-sensitizing effect of nMCA. Data are expressed as AUC of log RTS 14 days after treatment. (*) indicates a significant difference compared to the placebo control, $p < 0.05$, t-test., and (**) indicates a statistically significant difference compared to ionizing radiation only, $p < 0.05$, t-test; 1 Gy + 10 mg/kg nMCA, $p < 0.15$. The 1 Gy + 2 mg/kg data to the left (dotted bars), have been published earlier by Hua et al. 1995 (2).

transplanted into scid mice (2). In an attempt to improve the sensitized cytotoxic effect of combined treatment of radiation with nMCA (2 mg/kg; 1h pre-exposure), the radiation dose was increased to single doses of 6 and 10 Gy and combined with nMCA. However, even though the cytotoxic effect from increased radiation doses was elevated, the sensitizing effect from nMCA on radiation induced cytotoxicity was statistically insignificant at these radiation doses (Fig. 1 and Table 1).

To investigate if the results obtained in the H2981 tumor is a reflection of how radiosensitive the tumor is, the more radioresistant tumor A12B3 mouse sarcoma inoculated into CBA mice was also tested. Similar results were obtained in this tumor model where a relatively low radiation dose of 2 Gy was sensitized by nMCA at 2 mg/kg (Fig. 1 and Table 1). Moreover, at higher radiation doses of 6–18 Gy the additive effect from nMCA on A12B3 tumor growth reduction disappeared as was the case with H2981.

There was a clear tendency for the radiation induced cytotoxicity in the H2981 tumor to be increased at both the single doses of 2 and 10 mg/kg (Fig. 2 and Table 2). Hence, any differences between the 2 and 10 mg/kg nMCA doses were attributed to sample sizes rather than true statistical significance.

Toxicity. No acute toxic symptoms or death of animals were observed during the experiments. Yet, there was a slight but insignificant decrease in body weight the first

Table 1

The effect of nMCA (neutral metoclopramide) on radiation induced inhibition of tumor growth measured as specific growth delay (SGD) in human lung adenocarcinoma (H2981) xenographted into scid mice and a mouse sarcoma (A12B3) inoculated into CBA mice

	SGD ± SD[a]			
Dose[b]	n	XRT	n	XRT + nMCA
H2981 in scid				
1[c]	40	0.20 ± 0.43	46	0.45 ± 0.50[d]
6	11	2.02 ± 1.23	11	1.24 ± 0.88
10	11	3.11 ± 1.32	11	3.22 ± 0.72
A12B3 in CBA				
2	17	−0.05 ± 0.42	18	0.26 ± 0.43[d]
10	8	0.44 ± 0.56	7	0.56 ± 0.30
18	9	1.85 ± 2.01	11	1.72 ± 2.44

[a] Avg. ± S.D.
[b] The animals were exposed to radiation only (single dose) or in combination with nMCA (2 mg/kg, i.m.) administered 1 h before irradiation.
[c] Published earlier by Hua et al. 1995 (2).
[d] Students t-test, $p < 0.05$, XRT vs XRT + nMCA.

Table 2

The lack of effect of increased dose of nMCA (neutral metoclopramide) from 2 to 10 mg/kg on tumor growth of the lung adenocarcinoma (H2981) when combined with ionizing radiation

	SGD ± SD			
Treatment[a]	n	2 mg/kg[b]	n	10 mg/kg
Control	49	0.00 ± 0.45	17	0.00 ± 0.40
1 Gy	40	0.20 ± 0.43[c]	17	0.29 ± 0.48[c]
1 Gy + nMCA	46	0.45 ± 0.50[d]	19	0.55 ± 0.58[d]

[a] The animals were exposed to radiation (single dose) or radiation in combination with nMCA at 2 mg/kg or 10 mg/kg given 1 h before irradiation.
[b] Published earlier by Hua et al. 1995 (2).
[c] Students t-test, $p < 0.05$, XRT vs control.
[d] Students t-test, $p < 0.05$, XRT vs XRT + 2 mg/kg nMCA and $p < 0.15$, XRT vs XRT + 10 mg/kg nMCA.

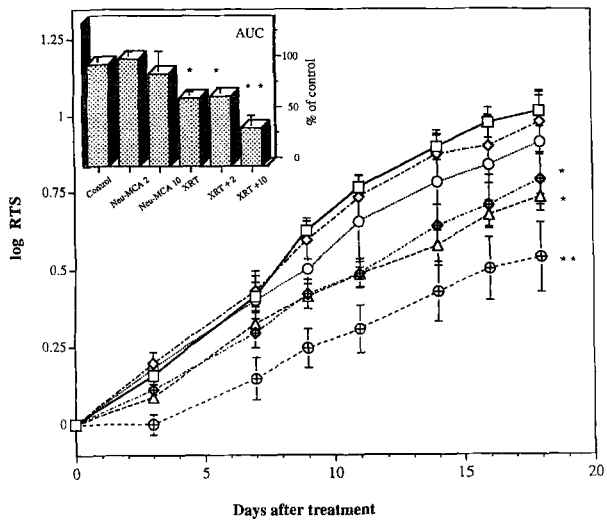

Fig. 3. Growth curve expressed as log RTS for the H2981 human lung adeno carcinoma xenografted into scid mice and treated with 3 fractionated doses of gamma radiation in combination with of nMCA at 2 or 10 mg/kg (-□-: control; -◇-: nMCA 3 × 2 mg/kg; ....○....: nMCA 3 × 10 mg/kg; --▽--: 3 × 1 Gy; -.◆.-: 3 × 1 Gy + nMCA 3 × 2 mg/kg; --⊕--: 3 × 1 Gy + nMCA 3 × 10 mg/kg). Inset shows area under the curve (AUC) of log RTS at day 14 after last treatment day. Data points represents mean ± s.e. and (*) indicates a statistically significant difference compared to the untreated control group and (**) indicates a significant difference compared to radiation only, $p < 0.05$, t-test.

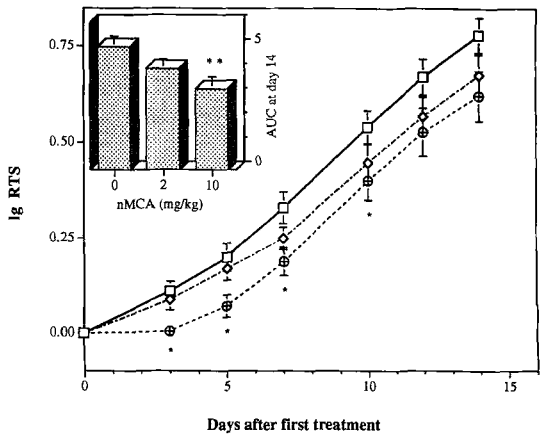

Fig. 4. Antitumor effect of nMCA (neutral metoclopramide) on the human lung adenocarcinoma (H2981) xenografted into scid mice after intramuscular administration of repeated doses at -□-: control, --◇--: 2 mg/kg and --⊕--: 10 mg/kg (i.m.; 8 doses; 1 dose every second day, starting day 7). Data points indicate mean ± s.e. Inset show AUC of the log RTS, mean ± s.e. (*) indicates a statistically significant difference in log RTS compared to the untreated placebo control group and (**) indicates a significant difference of AUC (inset) compared to the placebo control, $p < 0.05$, t-test.

week after start of treatment for all mice in all treatment arms (data not shown).

Repeated doses of MCA + radiation. When nMCA was administered in scid mice carrying the H2981 tumor as a fractionated dose of 3 × 10 mg/kg in combination with a fractionated radiation dose of 3 × 1 Gy (1 dose per day), the radiosensitizing effect by nMCA was statistically significant (Fig. 3 and Table 3, $p < 0.05$). In contrast there was no sensitizing effect when the nMCA dose was lowered and administered in the combined treatments of 3 × 2 mg nMCA/kg + 3 × 1 Gy radiation. These data, together with those reported in Figs. 1–2 or Tables 1–2, emphasize the importance of dose scheduling as well as the doses of radiation and nMCA to optimize the radiosensitizing effects.

Table 3

Dose fractionation effect of radiation when combined with nMCA (neutral metoclopramide) on H2981 tumor xenografted into scid mice. Results are expressed as tumor doubling time (TDt) and specific growth delay (SGD)

Treatment[a]	n	TDt[b]	n	SGD[b]
XRT	20	6.99 ± 3.90	20	0.40 ± 0.78
XRT + nMCA 2 mg/kg	10	7.64 ± 3.65	10	0.53 ± 0.73
XRT + nMCA 10 mg/kg	10	11.52 ± 7.90[c]	10	1.31 ± 1.59[c]

[a] The treatment was given as a fractionated dose (3 × 1 Gy; ± nMCA; 3 days).
[b] Average ± S.D.
[c] $p < 0.05$ compared to XRT only

Antitumor effect of nMCA. Since there seemed to be a tendency towards an antitumor effect from single doses of nMCA (Fig. 3), it was decided to increase the total dose of nMCA by repeated administrations using the H2981 tumor model and to determine the anti-tumor effects. H2981 tumor-bearing scid animals were thus treated with repeated doses (8 doses, given every second day) of nMCA at 2 and 10 mg/kg. This resulted in a dose dependent decrease in tumor growth ($p < 0.05$, ANOVA, Fig. 4). The anti-tumor effect of repeated doses (8 doses) of 10 mg/kg nMCA was mainly in the initial phase of the treatment with a statistically significant difference (t-test, $p < 0.05$) between the RTS values at day 3, 5, 7 and 10 after first treatment day (Fig. 4). The analysis of AUC of log RTS (day 14) showed a statistically significant difference ($p < 0.05$, Fig. 4, inset) at 10 mg/kg, but not at 2 mg/kg compared with the placebo control group. Further analysis of TDt and SGD also showed a significant effect at the repeated 10 mg/kg dose (Table 4). The anti-tumor effect on H2981 was not statistically significant when nMCA was given as 3 repeated doses, 1 × 3 on consecutive days, or when it was administered as a single dose at 2 and 10 mg/kg (Table 4).

The growth of the mouse sarcoma A12B3 was also significantly inhibited by repeated doses of nMCA (8 doses; every day) administered at 20 mg/kg (Table 4), but not at the lower doses of 2 and 10 mg/kg.

Table 4

Direct anti-tumor effect of nMCA (neutral metoclopramide) on human lung adeno-carcinomas (H2981) and mouse sarcoma (A12B3) expressed as specific growth delay

Treatment[b] mMCA	Dose (mg/kg)	n	Specific growth delay[a] Control Mean ± S.D.	n	nMCA Mean ± S.D.	Statistics[c]
H2981 tumor in scid mice						
single[d]	2	49	0.00 ± 0.45	18	0.05 ± 0.33	N.S.
single	10	18	0.00 ± 0.40	17	0.22 ± 0.60	N.S.
repeated 3 ×	2	15	0.00 ± 0.43	8	−0.09 ± 0.22	N.S.
repeated 3 ×	10	15	0.00 ± 0.43	8	0.25 ± 1.12	N.S.
repeated 8 ×	2	36	0.00 ± 0.53	23	0.17 ± 0.79	N.S.
repeated 8 ×	10	36	0.00 ± 0.53	28	0.47 ± 0.93	$p < 0.02$
A12B3 tumor in CBA mice						
repeated 8 ×	2	10	0.00 ± 0.26	9	0.05 ± 0.71	N.S.
repeated 8 ×	10	31	0.00 ± 0.45	28	0.03 ± 0.46	N.S.
repeated 8 ×	20	21	0.00 ± 0.29	20	0.44 ± 0.49	$p < 0.02$

[a] Calculated by SGD = (TDt − TDc)/TDc where TDt equals tumor doubling time for treatment group and TDc equals mean tumor doubling time for the control group.
[b] Scid mice were exposed to 2 or 10 mg/kg nMCA given as a single dose or doses repeated consecutively 3 × (daily) and 8 × (Monday, Wednesday, Friday, not weekends). CBA mice were given 8 repeated doses of 20 mg/kg (daily).
[c] Mann-Whitney U-Wilcoxon Rank Sum W Test.
[d] Published earlier by Hua et al. 1995 (2).

Apoptosis in the H2981 and A12B3 tumor lines. Because repeat low-dose radiation but not high single doses increased the radiosensitizing properties of nMCA, and because elevating the nMCA dose by repeated doses resulted in reduced tumor growth rate, it was reasoned that the drug may have targeted cytotoxic effects induced by apoptosis. In order to pursuit this reasoning further, we have determined the apoptotic constitutive fraction of cells in our H2981 and A12B3 tumor lines. The results reported in Table 5 clearly support the hypothesis that nMCA cytotoxic action is via induction of apoptosis. For example, A12B3 is more radioresistant to the cytotoxic effects of radiation and it has fewer constitutive apoptotic cells. In addition, A12B3 is also more resistant to the cytotoxic anti-tumor effects of nMCA administred in a repeat dose schedule (Table 4).

Table 5

Relationship between radiosensitivity, grade of apoptosis, growth rate and anti-tumor activity in the human lung adenocarcinoma (H2981) inoculated into scid mice and a mouse sarcoma (A12B3) in CBA mice

	H2981	A12B3
ED_{50} growth inhibition[a] (Gy)	7 Gy	18 Gy
Constitutive apoptosis[b] (%)	2.9 ± 0.5[c]	0.9 ± 0.3
Tumor doubling time (days)	5.2 ± 1.9	3.6 ± 0.7
Significant anti-tumor activity[d] (mg/kg)	10	20

[a] ED_{50} = The single dose of ionizing radiation (Gy) giving 50% reduction in tumor volume 5 days after treatment compared to sham irradiated tumors.
[b] Tumor sections stained with heamatoxylin and eosin; 400 × magnification (see Material and Methods).
[c] Mean ± S.D.
[d] nMCA administered as repeated dose (8 doses; Table 4)

Initial tumor volume effects. Our normal procedure has been to use initial tumor volumes of 60–200 mm^3 (4) but recently we have observed the fact that our results have become more consistent with initial tumor volumes of 50–75 mm^3 (2). As a consequence our laboratory has investigated this point in a more direct fashion and our data have indicated that nMCA was much more effective when the experiments were started with smaller tumor volumes (Table 5). Our results so far support the hypothesis that if the initial tumor volumes are <80 mm^3, the anti-tumor effects nMCA can be relied upon to show more optimal tumor responses when evaluated in our animal tumor models.

DISCUSSION

Neutralized MCA (nMCA) has been shown earlier to radiosensitize a human lung adenocarcinoma H2981 xenografted into scid mice at a relatively low (1 Gy) radiation dose (2). This study confirms the radiosensitizing effects reported earlier and further contributes three significant points for characterization of the mode of action of MCA, namely; a) The reduced side-effect profile of nMCA compared with generic acidic metoclopramide (8, 11) increased the feasability to elevate the dose of nMCA (from 2 mg/kg to 10 mg/kg or 20 mg/kg) which in turn resulted in

demonstration of enhanced radiosensitizing and anti-tumor effects (Figs. 2, 3 and 4, and Tables 2, 3 and 4), b) The radiosensitizing effect from nMCA was much improved when fractionated radiation was combined with nMCA (Table 2), and c) nMCA had an anti-tumor effect when it was administered by itself as repeated doses during the entire experiment (Fig. 4 and Table 4).

The mode of action of MCA is not fully understood, but several potential mechanisms have been identified: a) The DNA damage was significantly increased after radiation in presence of MCA both in vivo and in vitro (9, 10), b) MCA delayed DNA repair in vitro in mononuclear leukocytes (9) and in vivo in tumor tissue (10), c) MCA reacts with reduced glutathione after radiation which may lead to depletion of the glutathione pool and thereby the radiation effect on DNA damage and tumor cell toxicity would be enhanced (8) and finally d) the acidic and the neutral formulations of MCA induce apoptosis in the human leukemic cell line HL60 and the level of apoptosis was increased when combined by radiation (11). Taken together, these effects may at least in part explain why MCA has been shown to potentiate the radiation effects on tumor growth both in humans and in animal tumor models (2–7).

This study provides additional support to the hypothesis that nMCA controls tumor growth in vivo by inducing cytotoxicity by apoptosis in the following ways:

a) Low dose radiation but not high dose radiation is sensitized by nMCA (Fig. 1 and Table 1). These data suggest that the sensitizing mechanism could be via induction of apoptosis when combined with low doses of radiation (1–2 Gy) because low to moderate radiation doses (1–10 Gy) are known to increase the apoptotic fraction in tumor tissues (15, 16, 28). An explanation of the fact that nMCA did not sensitize at 6 to 18 Gy could be that at higher radiation doses the contribution from nMCA to induce cytotoxicity by apoptosis is relatively small, compared to the mitotic cell killing that occurs from the additional direct DNA damage that is introduced at higher radiation doses.

b) Repeat versus single dose radiation amplifies the nMCA sensitization response (Figs. 2–3 and Tables 2–3). Fractionated radiation doses (i.e. inverse split dose effect) have been shown to both increase the apoptotic fraction and reduce the clonogenic survival in tumor cells (29). Consequently, fractionated doses of a relatively low radiation dose given in combination with nMCA would be a more effective and clinically relevant (7) treatment regimen to increase the apoptotic fraction and thereby potentiate cytotoxicity of the tumor than the single dose treatment regimen.

c) High doses versus low doses of nMCA enhance the radiosensitizing and direct cytotoxic properities of this drug (Figs. 3–4 and Tables 3–4). One reason for nMCA's not directly giving anti-tumor effects at higher single doses (i.e. 10–20 mg/kg) whereas repeated doses do (Fig. 4 and Table 4), could be the fact that only a part of the tumor cells undergo apoptosis or are sensitive to apoptosis at any given time in the tumor (21, 30), due to which cell type, hierarchial status, phase of the cell cycle, or which oncogenes are being expressed in the tumor (16, 27, 30). Our recent in vitro data show that nMCA preferentially kills cells by apoptosis in a dose-dependent way at doses below 100 μM (11) which are comparable to a dose of about 32 mg/kg in vivo. Thus, if the nMCA dose in vivo is elevated from 2 to 10 mg/kg, a greater cytotoxic effect would be expected because optimal in vivo antitumor effects would not be reached below 32 mg/kg. Hence, elevating nMCA doses in single or repeated schedules would increase the tumor cytotoxicity of this drug since both 2 and 10 mg/kg doses are suboptimal for inducing maximum apoptotic effects. However, there are limitations caused by side-effects and toxicity of this drug at doses >32 mg/kg (1) and this fact needs to be taken into consideration for future clinical development.

Table 6

The effect of initial tumor volumes of human adenocarcinomas (H2981) xenografted into scid mice on the evaluation of inhibition of tumor growth by nMCA (neutral metoclopramide) alone

Initial tumor volume (mm^3)	AUC[a]				
	n	Control	n	nMCA[b]	t-test
≤80	20	5.23 ± 3.15	19	3.22 ± 2.78	$p < 0.04$
>80	10	4.10 ± 1.82	10	3.45 ± 5.95	$p = 0.51$

[a] Mean ± S.D.
[b] Repeated dose, 8 injections at 10 mg/kg (i.m.)

d) nMCA is more effective in treating tumors with higher constitutive levels of apoptosis. The slower growing tumor H2981 had a higher degree of spontaneous apoptosis compared with the mouse sarcoma A12B3 (Table 6). Moreover, estimating radiosensitivity by a 50% reduction in tumor volume 5 days after treatment (i.e. TCD_{50} values are not yet available), it was clearly shown that the H2981 tumor was more sensitive to ionizing radiation than the mouse sarcoma A12B3. The radiosensitivity was also correlated to growth rate and the constitutive level of apoptosis in the tumors (Table 5). In addition, the ability to inhibit growth of the tumor lines H2981 and A12B3 by repeated doses correlated well to the constitutive levels of apoptosis, radiosensitivity and growth rate of tumor tissue (Tables 4 and 5). Generally speaking it has been shown that tumor tissue homeostasis is influenced by the amount of apoptosis present where slow growing tumors have more apoptosis than fast growing ones (13). Thus, our data are in line with earlier reports by Meyn et al. (27), who have shown that there was a direct relation between the apoptotic response induced by radiation, radiosensitiv-

ity in terms of TCD50 and treatment efficacy. Moreover, the Meyn study also showed that tumors that were radiosensitive and displayed a high degree of spontaneous apoptosis also showed high levels of radiation-induced apoptosis.

e) nMCA is more cytotoxic against small tumors than large tumors (Table 6) which is an important aspect of the hypothesis that nMCA could induce apoptotic cell killing in vivo. When nMCA treatment was directed against smaller tumors, where the effect on the total viable tumor cell compartment would be greatest and where complications from direct induction of apoptosis by hypoxia are minimized (31), a repeated nMCA treatment was more effective at inducing tumor cytotoxicity presumably via an apoptotic mechanism. Our data also support the mode of action of nMCA being different from radiation and the two mechanisms being at least additive at lower radiation doses.

Additional support to the apoptosis model of radiation- and MCA-induced cytotoxicity is given by the study of Werning et al. (32), who showed that MCA sensitized a human squamous cell carcinoma xenographed into nude mice when MCA was combined with photodynamic therapy, an inducer of apoptosis (33). Studies to estimate the level of apoptosis in vivo and in vitro in relation to ionizing radiation and nMCA treatment are presently ongoing in our laboratory.

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