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Acidic and neutralized metoclopramide formulations sensitize ionizing radiation induced cytotoxicity in a human lung adenocarcinoma xenografted to scid mice

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A neutralized formulation (Neu-Sensamide) of metoclopramide (MCA) has been shown to possess reduced sedative side effects compared with the conventional acidic formulations (Primperan). The acidic formulation of MCA has also been shown to sensitize the effect of ionizing radiation (6–8 Gy) using human squamous cell carinomas from the head and neck xenografted to nude mice. In the present study, 2 mg MCA/kg body weight 1–3 h before treatment with 1 Gy radiation (single dose) was evaluated in scid mice xenografted with a human lung adenocarcinoma. MCA given alone in acidic or neutralized formulations did not show any effect on tumor growth retardation. However, when combined with radiation, both acidic and neutralized formulations of MCA sensitized the cytotoxic effect of radiation directed against the tumors by increasing tumor doubling time, tumor quadrupling time and specific growth delay, and by decreasing area under growth curve measurements. In addition, there was no statistically significant difference between the two formulations of MCA in the efficacy of sensitizing the cytotoxicity of a single low dose (1 Gy) of radiation.

Key words: Adenocarcinoma, metoclopramide, radiosensitization, scid mice, tumor growth.

Introduction

Metoclopramide (MCA) is a *N*-carboxamide substituted benzamide derivative. It has been evaluated as an effective clinical treatment of emesis induced by esophageal reflux or gastrointestinal disorders.[1] A high dose MCA (1–2 mg kg) was found to prevent emesis induced by chemotherapeutic drugs.[2] More recent studies have shown that MCA potentiated the effect of cisplatin on xenografted human squamous cell carcinomas (SCC) of the head and neck,[3,4] and sensitized the effect of ionizing radiation using the same animal model.[5] MCA also enhanced the cytotoxicity of BCNU [1,3-(2-chloroethyl)-1-nitrosourea] treatment of rat RG2 gliomas *in vivo* in the rat.[6]

The MCA drug formulations used in the above studies as well as in the clinic are offered in commercial form as acidic addition salts, pH = 2.0–4.5 (Lundbeck AB, Copenhagen; FASS 1993, Sweden), presumably because this form is freely soluble in aqueous solution whereas the free base form is quite water insoluble. However, our experiments in a rat model have shown that the acidic formulation of MCA caused a local tissue toxicity at the site of intramuscular injection and induced an extrapyramidal sedative side effect whereas a neutralized formulation of MCA induced no visible sedative symptoms at the same dose.[7] These results inspired us to investigate if commercial acidic (Primperan) or neutralized (Neu-Sensamide) versions of MCA could potentiate the effect of radiation in scid mice xenografted with another histopathologic type of non-small cell lung tumor, i.e. a human adenocarcinoma of the lung called H2981.

Radiation is a common therapy for treatment of cancer patients where its mechanism of action related to interaction with DNA, and so normal tissue could also be affected especially at high single doses of radiation.[8] Therefore, low doses of radiation per fraction below 2 Gy have been proposed in order to allow the total radiation dose to be raised without intolerable normal tissue damage.[9] In our previous studies, single doses of 5–8 Gy radiation were used and they were sensitized by MCA using xenografted human SCC.[5] In this study, 1 Gy radiation was tried to see if MCA could sensitize an even more clinically relevant dose of radiation.

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Materials and methods

Mice

Six-week-old scid mice of both sexes were used. The weights of animals were 20–25 g for female mice and 25–30 g for male mice. They were maintained under sterile but not specific-pathogen-free conditions.

Tumor line

Tumor line H2981 used in these experiments originated from a human lung adenocarcinoma that has been established in *in vitro* cell culture[10] and implanted into scid mice. The tumor grafts were serially transplanted by subcutaneous inoculation of human lung carcinoma tissue suspension into the right flank of mice for 20–25 generations.

Drug treatment

MCA [4-amino-*N*-(2-diethylaminoethyl)-5-chloro-2-methoxy-benzamide] was obtained as a commercially available 100 mg/ml sterile infusion concentrate, pH = 2.0–3.5 (Primperan®; Lundbeck AB, Copenhagen, Denmark). Neutralized metoclopramide (Neu-Sensamide®) was provided as a 100 mg/ml sterile injectable concentration, pH = 6.7–6.9, by OxiGene (New York). Both chemicals were diluted with physiologic saline and sterilized by filtration at a concentration of 1–2 mg/ml. They were given by intramuscular injection in a volume of 50 μl which corresponded to 2 mg/kg, a dose used in the clinic. Other animals were given saline in equivalent volume as a placebo control. The animals were monitored for acute symptoms throughout the evaluation period.

Irradiation

Radiation with a ^{60}Co γ-ray source (4.76 Gy/min) was given to the animals 1–3 h after MCA injection. The tumors were locally irradiated with a single dose of 1 Gy while the mice were under anaesthesia (Sombrevin®).

Tumor volume measurement

All tumors were scored with a calliper after 1 week of inoculation. The tumor volumes were calculated as: volume = $(L \times W^2)/2$, where L is the length of tumor and W is the width of the tumor. The tumor volume according to this formula correlates well with exercised tumor weight. When the average tumor volume reached 50–70mm^3, the animals were randomly divided into several groups which insured no significant difference of sex, body weight and initial tumor volumes between the groups. The tumors were measured every other day after treatment until the time tumors developed necrosis or they reached a perpendicular axis measurement of 15 mm × 15 mm, which was usually 13 days after treatment.

Evaluation of tumor response

Relative tumor size (RTS) was calculated as tumor volume at the time of measurement divided by tumor volume at the start of treatment. To obtain a normal distribution of RTS values, the $\log_{10}$ of RTS was used and the growth curve of the log RTS versus time was plotted. Two indicators were used to evaluate treatment efficacy: the area under growth curve (AUC) and specific growth delay (SGD). SGD was calculated according to Berman and Steel.[11] The growth curves fitted by a polynomial regression were used to calculate the time taken for each tumor to grow to double its size (DT = tumor doubling time) or four times its size (QT = tumor quadrupling time) relative to tumor volume measurment at the start of treatment. The growth delay (GD) was calculated as the difference between the DT of each individual tumor and the mean DT for the control tumors ($GD = DT_t - DT_c$). SGD was calculated as $SGD = (DT_t - DT_c)/DT_c$. SGD can be regarded as the number of DT gained by the treatment. In order to avoid potential pitfalls, we analyzed the growth curves of individual tumors rather than the mean of each treatment group. The area under curve (AUC) was calculated from the growth curve of log RTS versus time according to Lesser *et al.*[12] AUC gives an overall index of tumor size, and accounts for both degree and duration of inhibition. Daily fluctuations will be smoothed out by this calculation. The observation period was set to 13 days after treatment.

Statistics

Data analysis was carried out using the SPSS program (SPSS Inc.). Differences between groups were

tested by one-way analysis of variance (ANOVA) and differences between two groups were further analyzed by the Duncan test at the significant level of 0.05.

Results

Figure 1 depicts log RTS measurements plotted in relation to treatment time. Each point on the curve represents the mean log RTS of all mice in that particular treatment group for that particular time. Tumors from mice treated with radiation alone showed a slower growth when compared with those for the placebo-treated controls. Tumors treated with the acidic or neutral form MCA in combination with radiation grew even more slowly, whereas tumors administered with MCA alone either in acidic or neutral forms did not show growth delay when compared to tumors of placebo-treated controls.

Further analysis of growth delay (Table 1) showed that the values of tumor doubling time, tumor quadrupling time and SGD for both acidic or neutral MCA alone were not significantly different from those of the placebo-treated controls. Both formulations of MCA did not significantly inhibit or stimulate tumor growth. However, when tumors treated with a combination of MCA and radiation were considered, there was a significant difference not only when compared with placebo-treated controls or with the MCA treatment groups alone, but also with the radiation alone treated group. For the group treated with neutral MCA in combination with radiation, significant differences were manifested as a longer doubling and quadrupling time and higher SGD ($p < 0.05$ versus radiation treatment). For the group treated with acidic MCA in combination with radiation, the significant difference was observed in the index of tumor quadrupling time ($p < 0.05$ versus radiation treatment) indicating a more delayed effect on tumor growth. Taken together these results demonstrate that both acidic and neutral MCA sensitized the effect of radiation on tumor growth. However, there were no statistically significant differences between acidic and neutral MCA either when given alone or in combination with radiation.

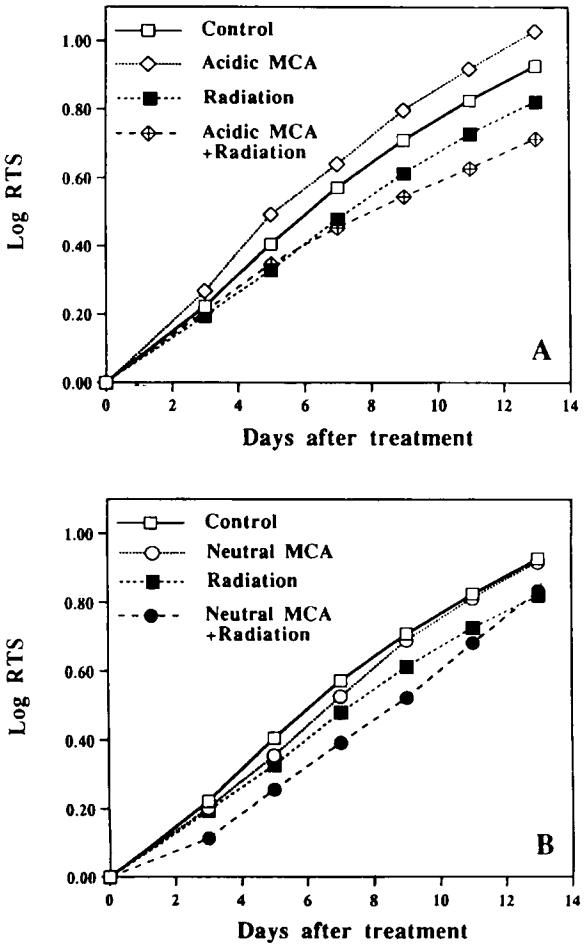

Figure 1. Growth curves of H2981 human lung adenocarcinoma in scid mice treated with the acidic form MCA (A, Primperan) and the neutral form MCA (B, Neu-Sensamide). The curves are plotted in log RTS over time. Each point is the mean of between 17 and 49 animals.

Table 1. Values of tumor doubling time, tumor quadrupling time and SGD for H2981 human lung adenocarcinoma in scid mice treated with radiation, MCA or the combination of two

Treatment	Number	Tumor doubling time (days)	Tumor quadrupling time (days)	SGD
Controls	49	3.81±1.71	7.86±2.48	0.00±0.45
Acidic MCA	17	3.18±0.70	6.73±1.31	−0.17±0.18
Neutral MCA	27	4.00±1.27	8.15±1.98	0.05±0.33
Radiation	40	4.55±1.62[a]	8.92±2.26[a]	0.20±0.43[a]
Acidic MCA + radiation	28	4.81±1.89[a,c]	10.87±2.99[a,b,c]	0.28±0.49[a,c]
Neutral MCA + radiation	46	5.53±1.90[a,b,c]	9.88±2.20[a,b,c]	0.45±0.50[a,b,c]

[a] $p < 0.05$ versus controls; [b] $p < 0.05$ versus radiation alone; [c] $p < 0.05$ versus acidic or neutral MCA alone.

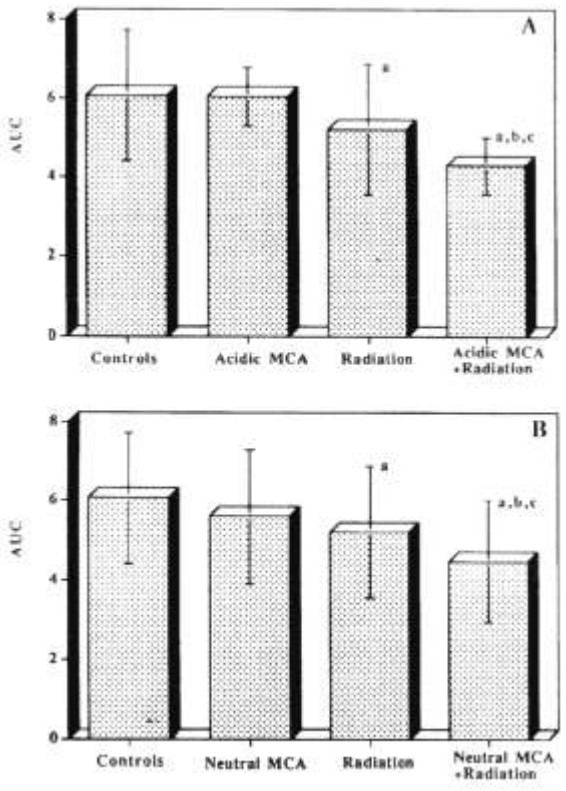

Figure 2. AUC values for the tumor line H2981 in scid mice treated with radiation, MCA and both in combination. (A) A study of the acidic form of MCA (Primperan). (B) A study of the neutral form MCA (Neu-Sensamide). The data are means ± SD. $^{a}p < 0.05$ versus controls; $^{b}p < 0.05$ versus radiation; $^{c}p < 0.05$ versus acidic or neutral MCA alone.

The above results were also confirmed by the analysis of AUC (Figure 2), a powerful statistical index evaluated in this case after 13 days of treatment. When the tumors were given radiation alone, the AUC values were 80% of the placebo-treated controls. However, when treated with MCA in combination with radiation, the value was significantly reduced to 70% of the placebo-treated controls for acidic MCA and 71% for neutral MCA, whereas when MCA was administered alone either in acidic or neutral form, the values showed no statistically significant reduction when compared with the placebo-treated controls. Moreover, there was no significant difference between the two formulations of MCA in AUC analyses when the drugs were given alone or in combination with radiation.

Toxicity was evaluated as the appearance of acute symptoms over time. There were no differences between the various treatment groups in regard to the development of symptoms that could lead to death or sacrifice of the animal.

Discussion

Earlier studies have shown that MCA sensitized the cytotoxicity of radiation or cisplatin of human SCC which were xenografted into immune deficient nude mice.[3,5] The present study used T cell deficient scid mice xenografted with a human lung adenocarcinoma to evaluate the radiosensitizing properties of MCA administered at the same dose (i.e. 2 mg/kg body weight) and schedule as was used in the above-mentioned studies. However, the earlier experiments were carried out using intraperitoneal injection of the drug whereas the experiments reported here used intramuscular injection. The data indicated that (i) MCA at 2 mg/kg did not induce any obvious toxic symptoms, which was in agreement with the previous reports,[3,5] and (ii) MCA sensitized the effect of radiation of another histopathologic type of human cancer, a lung adenorcarcinoma. These results broaden the potential clinical application of MCA as a sensitizer of radiation.

The MCA formulation used in our previous studies was provided as an acidic addition salt (pH = 2.0–4.5, Primperan), presumably because this form is freely soluble in aqueous solution. However, acidic MCA caused a local tissue toxicity at the site of intramuscular injection and induced an extrapyramidal sedative side effect whereas a neutralized formulation of MCA (Neu-Sensamide) induced no visible symptoms when administered at the same dose.[7] Our chemical explanation is that there is a hydrogen bond formed in acidic MCA addition salts between the carbonyl of the *N*-substituted amide of benzamide and the proton on the nitrogen of the dimethylaminopropyl side chain which helps planarize the molecule and allows binding to the dopamine (D_2) receptor.[7] It is well documented that planarization of the MCA structure is essential for binding to the dopamine (D_2) receptor.[13] Therefore, the question was raised whether or not neutralized MCA could have less extrapyramidal side effects as reported above and still be able to maintain its radiosensitizing properties. The present data clearly support that neutralized MCA at the same dose as acidic MCA and in combination with radiation sensitized the cytotoxicity induced in adenocarcinomas by radiation to a comparable degree. To our knowledge this is the first report of neutral MCA as a radiosensitizer. Because of the reduced side effects

of neutral MCA, this drug may be a superior version of MCA for further development in the clinic as a radiosensitizer.

The mechanism behind the radiosensitizing effects of MCA is not quite clear. It has been postulated that MCA sensitizes radiation and chemotherapy by two mechanisms; namely by increasing DNA damage or inhibiting DNA repair or a combination of both ways. Studies have demonstrated that MCA interfered with the DNA repair process[14] and it also could increase the activity of poly[adenosine diphosphote ribosyl] transferase (polyADPRT), an enzyme requiring DNA strand breaks for activity.[15] Increases in DNA damage *in vitro* in human mononuclear leucocytes and *in vivo* in mouse tumor tissue were observed in the treatment of MCA combined with radiation compared with treatment with radiation alone.[14,16] The DNA damage and repair inhibition could be due to MCA reactive products induced by radiation. In this regard, it was shown that MCA in aqueous solution decomposed into several products after radiation and one of these products was able to react with the reduced form of glutathione.[7] These preliminary data suggest that the radiosensitizing mechanism of MCA may involve depletion of cellular glutathione pools or chemical interaction with a thiol-containing DNA repair enzyme such as polyADPRT. These mechanisms would inhibit DNA repair and increase DNA damage which in turn would lead to increased cytotoxicity. Further studies are underway in our laboratory to substantiate if irradiated products of MCA are involved in mediating radiosensitizing properties of the drug.

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References

1. Harrington RA, Hamilton CW, Brogden RN, *et al.* Metoclopramide. An updated review of its pharmacological properties and clinical use. *Drugs* 1983; **25**: 451–94.
2. Gralla RJ, Tyson LB, Clark RA, *et al.* Antiemetic trials with high dose metoclopramide: superiority over THC, and preservation of efficacy in subsequent chemotherapy courses. *Proc Am Soc Clin Oncol* 1982; **23**: 58.
3. Kjellén E, Wennerberg J, Pero RW, Metoclopramide enhances the effect of cisplatin on xenografted squamous cell carcinoma of the head and neck. *Br J Cancer* 1989; **59**: 247–50.
4. Lybak S, Wennerberg J, Kjellén E, *et al.* Dose schedule evaluation of metoclopramide as a potentiator of cisplatin and carboplatin treatments of xenografted squamous cell carcinomas of the head and neck. *Anti-Cancer Drugs* 1991; **2**: 375–82.
5. Lybak S, Kjellén E, Wennerberg J, *et al.* Metoclopramide enhances the effect of ionizing radiation on xenografted squamous cell carcinoma of the head and neck. *Int J Radiat Oncol Biol Phys* 1990; **19**: 1419–24.
6. Salford LG, Pero RW, Aas AT, *et al.* Metoclopramide as a sensitizer of 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) treatment of brain tumors in the rat. *Anti-Cancer Drugs* 1992; **3**: 267–72.
7. Pero RW, Olsson A, Sheng Y, *et al.* Progress in identifying clinical relevance of inhibition, stimulation and measurement of poly ADP-ribosylation. *Biochimie* 1995; in press.
8. Baverstock KF, Will S. Evidence for the dominance of direct excitation of DNA in the formation of strand breaks in cells following irradiation. *Int J Radiat Biol* 1989; **55**: 563–8.
9. Horwich A. Cancer research campaign review of radiation research. *Br J Cancer* 1993; **67**: 198–201.
10. Schreiber GJ, Hellström KE, Hellström I. An unmodified anticarcinoma antibody, BR96, localizes to and inhibits the outgrowth of human tumors in nude mice. *Cancer Res* 1992; **52**: 3252–66.
11. Berman R, Steel GG. Induced and inherent resistance to alkylating agents in human small-cell bronchial carcinoma xenografts. *Br J Cancer* 1984; **49**: 431–6.
12. Lesser ML, Braun HL, Helson L. Statistical methods for measuring and comparing treatment efficacies: application to nude mice experiment. *Exp Cell Biol* 1980; **48**: 126–37.
13. Anker L, Testa B, Waterbeemd H, *et al.* A basicity, lipophilicity and lack of receptor interaction of *N*-aminoalkylbenzamides and *N*-aminoalkyl-*o*-anisamides as model compounds of dopamine antagonists. *Helv Chim Acta* 1983; **66**: 542–54.
14. Lybak S, Pero RW. The benzamide derivative Metoclopramide causes DNA damage and inhibition of DNA repair in human peripheral mononuclear leukocytes at clinically relevant doses. *Carcinogenesis* 1991; **12**: 1613–7.
15. de Murcia G, Ménissier-de Murcia J, Schreiber V. Poly (ADP-ribose)-polymerase: molecular biological aspects. *BioEssays* 1989; **13**: 455–62.
16. Olsson A, Sheng Y, Kjellén E, *et al.* *In vivo* tumor measurement of DNA damage, DNA repair and NAD pools as indicators of radiosensitization by metoclopramide. *Carcinogenesis* 1995; **16**: in press.

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