Anti-Cancer Drugs, 3, pp. 267–272

Metoclopramide as a sensitizer of 1,3-bis(2-chloroethyl)-1-nitrosourea treatment of brain tumors in the rat

Leif G Salford, Ronald W Pero[CA], Alf T Aas and Arne Brun

AT Aas and LG Salford are at the Division of Experimental Neurooncology, Department of Neurosurgery, University of Lund, S-221 85, Lund, Sweden. A Brun is at the Division of Neuropathology, Department of Pathology, University of Lund, S-221 85, Lund, Sweden. RW Pero is at the Department of Molecular Ecogenetics, The Wallenberg Laboratory, University of Lund, S-220 07, Lund, Sweden.
Tel: (46) 46 107000. Fax: (46) 46 104624.

RG2 glioma-like cells grown in *in vitro* culture can be inoculated into rat brains using stereotactic surgical procedures to produce tumors with a diameter of 12–16 mm^2 in 20–21 days. This system has been used to evaluate if metoclopramide (MCA) could sensitize the tumor toxicity of 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU). BCNU alone (15 mg/kg, intravenously), and MCA alone (2 mg/kg, intraperitoneally), and these drug treatments in combination, were administered so that BCNU alone was given as a single dose on day 3 after inoculation of the RG2 cells, MCA alone was given on day 3 at 0 and 3 h followed by five or six treatments per week beginning 24 h after the 3 h dose, and BCNU plus MCA were given according to the combined schedule where the first MCA treatment was scheduled 30 min prior to the BCNU infusion. The design of this study required the drug treated animals to be matched to untreated animals (controls) at the time of inoculation of the RG2 cells. Under these experimental conditions, BCNU alone and MCA alone had no effect on tumor growth, whereas BCNU plus MCA significantly retarded brain tumor growth. The normal tissue toxicity induced by BCNU treatment, evaluated by measurement of body weight and survival, was not potentiated by the combination of BCNU plus MCA. These data extend the previous findings of MCA as a radio- and chemosensitizer to include the sensitization of another cytotoxic agent (BCNU) and of another type of tumor (malignant glioma).

Key words: Brain tumor, 1,3-bis(2-chloroethyl)-1-nitrosourea, metoclopramide, sensitizer.

Introduction

There are over 20 000 new cases of malignant tumors of the central nervous system diagnosed each year in the USA. Despite multimodality therapy, the prognosis for these patients is very poor with mean survival ranging between 3 months and 1 year.[1] One primary factor contributing to the poor prognosis of brain tumor patients is the lack of efficacy of conventional chemotherapeutic drug treatments, because many possess inappropriate pharmacodynamics to cross the blood–brain barrier. Therefore, there is a great need to develop new approaches that will overcome the current limitations in drug development for brain tumor control. In this regard our approach has been to consider drug combinations that might sensitize the chemotherapeutic agents showing some limited success in the clinic today.

The most conventional chemotherapies given to brain tumor patients are the class of drugs that can be chemically characterized in a general way as nitrosoureas. 1,3-Bis(2-chlorethyl)-1-nitrosourea (BCNU) is the most common nitrosourea given to the patients, where more than 60% fail to respond and, therefore, they require some form of drug resistance therapy.[1–3] Our laboratories have been interested in developing a brain tumor model in the rat, so that comparisons between conventional BCNU chemotherapy and promising new treatment modalities can be carried out. This has been accomplished by inoculating RG2 rat glioma-like cells[4] into the brain of rats by using stereotactic surgical procedures and then histopathologically evaluating tumor growth.

Metoclopramide (MCA) is an N-carboxamide substituted benzamide that is currently being used in the clinic as an antiemetic.[5,6] MCA antagonizes

[CA] Corresponding Author

the dopamine-2 receptor[5] and, hence, it is known to cross the blood–brain barrier. MCA has also been shown to be an effective sensitizer of both radiation[7,8] and cisplatin[9,10] therapies when evaluated against human squamous cell carcinomas xenografted into nude mice. MCA did not enhance normal tissue toxicity[7-10] and it was effective at doses currently given in the clinic as an antiemetic.[11,12] There have been at least two modes of action identified for MCA that could explain at the molecular level its ability to sensitize cytotoxic drugs, i.e. inhibition of DNA repair and direct induction of DNA damage.[13] The properties of MCA, such as its ability to cross the blood–brain barrier and to sensitize cytotoxic drugs at clinically relevant doses, have indicated to us the potential for its combination with BCNU to sensitize the toxicity directed toward brain tumors. Here, we report that MCA given in combination with BCNU gave greater toxicity against brain tumors in an *in vivo* rat model than either drug did given separately.

Materials and methods

Rats

Male and female Fischer 344 rats aged between 10 and 12 weeks and weighing 150–200 g were used. All animals exhibited progressive weight gain at the initiation of each experiment. Groups of 10–12 animals were paired so that drug treated animals always had a matched control designated at the time of inoculation of tumor cells.

Tumor

The RG2 rat glioma cell line, which was established in *in vitro* culture over 20 years ago from a ethylnitrosourea induced glioma-like rat tumor, was used for these studies.[4] About 3000 RG2 cells, harvested from *in vitro* culture in log phase growth, were injected in a 5 μl volume of culture medium into the head of the right caudate nucleus using stereotactic surgical procedures. Well delineated tumors developed in 100% of the animals within 21 days at which time the tumors reached a diameter of about 3–6 mm.

Tumor growth measurements

Whenever RG2 glioma cells were inoculated into rats in groups of 10–12 animals and they began to develop neurological symptoms (i.e. after 20–21 days), all animals within the group were sacrificed by perfusion-fixation of the brains under chlorahydrate anesthesia. All brains were examined histopathologically by one of us (A.B.). Five coronal slices from each rat brain were studied microscopically using crystal violet staining. In this way, the whole telencephalon of the brain was covered except for the frontal and occipital poles. The slice with the largest tumor portion was chosen for a simplified calculation of tumor size. The largest diameter (mm) was multiplied by its perpendicular diameter (mm) resulting in the estimate of tumor size surface as a square (mm^2). No attempts were made to adjust for the exact form of the tumor as the values of interest were always paired between drug treated animals and their controls.

Drugs

Both MCA and BCNU were obtained from the Lund Hospital pharmacy as commercially available preparations for human use. MCA was supplied as Primperan (A Lundbeck A/S, Copenhagen, Denmark) in a 5 mg/ml solution. This solution was diluted to 0.5 mg/ml with sterile saline and injected intraperitoneally at a dose of 2 mg/ml. BCNU was supplied as Carmustine (Bristol Myers) in 100 mg ampoules. Carmustine was dissolved in 3 ml 95% ethanol and then diluted with sterile water to 3.3 mg/ml. The Carmustine (BCNU) solution was administered intravenously through the femoral vein at a dose of 15 mg/kg. The BCNU solutions were made up fresh just prior to injection.

Dose schedule

The dose schedule for BCNU (15 mg/kg) alone was a single administration of the drug on day 3 after inoculation of the rats with the RG2 glioma cells. MCA (2 mg/kg) alone was also given on day 3 followed by an additional 2 mg/kg dose given 3 h later, and then additional 2 mg/kg doses beginning 24 h later and being repeated five or six times per week for 20–21 days. The combined MCA plus BCNU dose schedule was the same as described for each drug separately where the first MCA intraperitoneal injection was started 30 min prior to the intravenous infusion of BCNU.

Toxicity

The toxicity of the various treatment regimens involving MCA, BCNU or the drugs in combination was assessed by changes either in survival or in the body weights of surviving animals.

Statistics

Because the experimental design of our study requires matching a control animal with a drug treated animal at the time of inoculation of the RG2 glioma cells by stereotactic surgical procedure, we have used the paired Student's *t*-test as the appropriate statistical approach. When comparing the various matched control groups or the drug treated groups with each other, the unpaired Student's *t*-test was considered appropriate.

Results

MCA sensitization of BCNU tumor toxicity

Preliminary BCNU dose–response data were collected using the RG2 brain tumor model in the rat in order to select a dose of BCNU that would be just below the concentration which gives significant reductions in tumor growth. This dose was found to be 15 mg/kg (data not shown). Using this standardized dose of BCNU, *in vivo* comparisons of rats treated with BCNU alone, MCA alone, in combination (BCNU plus MCA) or untreated controls, were carried out. MCA was used at a dose of 2 mg/kg because this dose is routinely given in the clinic to prevent emesis[5,6,11,12] and it is also known to sensitize the cytotoxicity induced by both cisplatin or radiation.[7–10] The pertinent data are presented in Table 1. The paired design of this study has allowed us to show that neither BCNU alone ($n = 31$) nor MCA alone ($n = 14$) had any significant effect on tumor growth when compared directly with their matched controls. However, the combination of BCNU plus MCA did give a significant reduction ($p < 0.004$) in tumor growth compared with matched controls. These data clearly indicate that MCA sensitized the cytotoxic action of BCNU directed against RG2 glioma cells grown *in vivo* in the rat. There is also included an unpaired analysis of the data in Table 1. Firstly, there were no significant differences between the various control groups, indicating that this particular RG2 brain tumor model in the rat is quite reproducible from one group of animals to another. Secondly, intergroup comparisons have revealed that only the BCNU plus MCA group was significantly different from all the other treatment groups.

Tumor growth effects on MCA sensitization

The RG2 glioma model in the rat requires inoculation and subsequent growth of brain tumors without the possibility of evaluation until the animals are sacrificed at day 20–21 (see Materials

Table 1. Comparison of the *in vivo* tumor toxicity of BCNU, MCA and BCNU plus MCA in Fischer rats transplanted with the RG2 glioma line (3000 cells) for 20–21 days

Treatment	Drug dose (mg/kg)	*n*	Tumor size [mean (mm^2) ± SD]	Paired Student's *t*-test analysis		Unpaired Student's *t*-test analysis	
				groups	significance	groups	significance
1 BCNU only	15	31	13.7 ± 10.5	—		1 to 5 1 to 2, 3, 4, 6	$p < 0.004$ NS
2 Matched controls	0	31	16.0 ± 11.3	1 to 2	NS	2 to 5 2 to 3, 4, 6	$p < 0.001$ NS
3 MCA only	2	14	12.8 ± 8.4	—		3 to 5 3 to 4, 6	$p < 0.007$ NS
4 Matched controls	0	14	12.8 ± 7.1	3 to 4	NS	4 to 5 4 to 6	$p < 0.001$ NS
5 BCNU plus MCA	15 + 2	27	7.4 ± 3.9	—		5 to 6	$p < 0.001$
6 Matched controls	0	27	13.0 ± 9.1	5 to 6	$p < 0.004$	—	—

and methods). Although the data in Table 1 support the conclusion that brain tumors can be grown *in vivo* in a fairly uniform way, there is the problem of determining if day 3 after inoculation is sufficient time to allow the effects of drugs on tumor growth to be assessed. In order to gain insight into this problem, we have divided the control groups into two groups, e.g. one group with small brain tumors (< 10 mm^2) and the other group with larger brain tumors (≥ 10 mm^2), and then these groups were compared for brain tumor size with their matched drug treated animals. The data presented in Table 2 demonstrate that when the tumors were small at the time of sacrifice (< 10 mm^2), indicating poor tumor growth during the evaluation period of 20–21 days, no effect of MCA plus BCNU on tumor growth could be observed when compared with matched controls. However, when the matched controls had larger tumors at the time of sacrifice, indicating good tumor growth during the evaluation period of 20–21 days, there was a highly significant reduction ($p < 0.001$) in tumor size in the BCNU plus MCA treated group compared with the matched control group. These data show that there is considerable variation in the growth of RG2 glioma cells after 20–21 days in the brain of rats, so that when the cells grow into tumors smaller than 10 mm^2, they are insensitive in evaluating the tumor toxicity of drugs. This is true when the extremes in variability in tumor growth rate become equal in magnitude to the cytotoxic effects of the drug. Nonetheless, this analysis of the data still confirms that BCNU brain tumor toxicity is sensitized by MCA.

Toxicity

The RG2 brain tumor model in the rat employs stereotactic surgery on every specimen. All animals survived the treatments and lived through the observation period of 20–21 days. In Table 3, it can be seen that this procedure was undoubtedly dramatic to the rats, since even the control animals lost weight during the 3 week observation period. Moreover, both the doses of BCNU (15 mg/kg) and MCA (2 mg/kg) used in this study were toxic to the animals, since the animals in these groups lost even more weight than did the untreated controls. However, the BCNU plus MCA combination did not potentiate the weight loss over that observed for BCNU alone, indicating that MCA did not enhance the normal tissue toxicity of BCNU.

Table 2. The effect of initial tumor size, estimated in untreated rats inoculated with 3000 RG2 glioma cells for 20–21 days, on the *in vivo* enhancement of BCNU tumor toxicity by MCA

Treatment	Drug dose (mg/kg)	*n*	Tumor size [mean (mm^2) ± SD]	Paired Student's *t*-test analysis	
				groups	significance
1 BCNU only	15	10	8.5 ± 6.2		
2 Matched controls tumors < 10 mm^2	0	10	4.9 ± 3.0	1 to 2	NS
3 BCNU only	15	21	16.2 ± 11.2		
4 Matched controls tumors ≥ 10 mm^2	0	21	21.2 ± 11.2	3 to 4	NS
5 MCA only	2	4	15.4 ± 11.1		
6 Matched controls tumors < 10 mm^2	0	4	6.3 ± 2.1	5 to 6	NS
7 MCA only	2	10	9.0 ± 6.3		
8 Matched controls tumors ≥ 10 mm^2	0	10	15.1 ± 7.2	7 to 8	NS
9 BCNU plus MCA	15 + 2	12	6.3 ± 2.5		
10 Matched controls tumors < 10 mm^2	0	12	5.3 ± 2.1	9 to 10	NS
11 BCNU plus MCA	15 + 2	14	8.5 ± 4.7		
12 Matched controls tumors ≥ 10 mm^2	0	14	19.7 ± 7.4	11 to 12	$p < 0.001$

Table 3. The effect of *in vivo* exposure to BCNU, MCA and BCNU plus MCA on the body weights of rats inoculated in the brain with 3000 RG2 cells for 20–21 days

Treatment	drug dose (mg/kg)	*n*	Weight reduction [mean (g) ± SD]	Unpaired Student's *t*-test analysis	
				groups	significance
1 Controls	0	60	8 ± 19	—	
2 MCA only	2	14	20 ± 14	1 to 2	$p < 0.03$
				2 to 3	$p < 0.007$
				2 to 4	$p < 0.001$
3 BCNU only	15	28	40 ± 24	1 to 3	$p < 0.0001$
				3 to 4	NS
4 BCNU plus MCA	15 + 2	21	39 ± 17	1 to 4	$p < 0.0001$

Discussion

This study has shown that MCA can sensitize BCNU cytotoxicity directed against brain tumors evaluated *in vivo* in the rat (Tables 1 and 2). This was accomplished without any significant potentiation of normal tissue toxicity above that observed for BCNU alone (Table 3). These results are encouraging for the clinical development of more successful treatment regimens for malignant glioma. As already mentioned, MCA has known activity on the central nervous system and the dose used here to sensitize (2 mg/kg) is already given daily in the clinic to treat emesis.[5,6,11,12] Because BCNU chemotherapy is known to be effective against human malignant gliomas,[1-3] there is a strong logical basis to consider taking this therapeutic approach to clinical trials.

The normal administration schedule for MCA as an antiemetic is at doses of 1–3 mg/kg delivered intravenously and repeated every 2 h up to a total of three to five doses every 24 h to prevent nausea and vomiting induced by chemotherapeutic drugs.[11,12] MCA as a sensitizer of cisplatin and radiation therapies was administered at 2 mg/kg intraperitoneally at the time of cisplatin or radiation therapy, and 24 and 48 h after delivery of the cytotoxic agent.[7-10] There was no evidence in either the earlier human studies or the animal tumor model studies that MCA potentiated the normal tissue toxicity of either cisplatin or radiation. Moreover, MCA alone given at 0, 24 and 48 h did not influence weight gain or survival, indicating a lack of toxicity to normal tissues.[7-10] This is in contrast to this study where MCA alone induced a significant increase in weight loss when compared with untreated controls (Table 3). However, MCA was administered not only in a similar repeated dose schedule for the first 48 h but 5–6 times per week for 20–21 days. Because MCA has never been given in such a prolonged repeated dose schedule before, these data could be taken as evidence of MCA induced normal tissue toxicity. This point needs further clarification in the future.

This study also broadens the application of MCA as a sensitizer of cytotoxic agents. Previously, MCA had only been shown to sensitize the killing of squamous cell carcinomas, but now we know that other tumor types such as gliomas can also be killed by a MCA sensitizing mechanism. In addition, BCNU can be added to the list of cytotoxic agents (i.e. cisplatin and radiation) whose action is sensitized by MCA. BCNU, cisplatin and radiation are believed to kill tumor cells because treatment with these agents results in increased cellular DNA damage that can inhibit cell replication, which in turn can lead to cell death. MCA must in some way enhance the DNA damage induced by these agents. There are only two primary ways a sensitizer can increase DNA damage: (i) it can interact with the DNA damaging process induced by the cytotoxic agent or (ii) it can interfere with the DNA repair process resulting in the accumulation of DNA damage (or a combination of both ways). MCA has been shown to possess both these sensitizing mechanisms,[13] which probably explains its versatility and effectiveness as a radio- and chemosensitizer. For example, the extensively studied nitroimidazoles are a class of sensitizer where multiple modes of action have not been identified[14,15] and despite over 30 years of research these agents have not reached the clinic.

Acknowledgments

This study was supported by the Swedish Cancer Society, Crafoord Foundation, King Gustaf V Jubilee Fund and the Medical Faculty of Lund University, all located in Sweden, and Oxigene Inc., New York, NY, USA.

References

1. Green SB, Byar DP, Walker MD *et al.* Comparisons of carmustine, procarbazine, and high-dose methyl-prednisolone as additions to surgery and radiotherapy for the treatment of malignant glioma. *Cancer Treat Rep* 1983; **67**: 121–32.
2. Walker MD, Hurwitz BS. BCNU (1,3-bis(2-chloro-ethyl)-1-nitrosourea; NSC 409962) in the treatment of malignant brain tumors. A preliminary report. *Cancer Chemother Rep* 1970; **54**: 263–71.
3. Wilson CB, Boldrey EB, Enot KJ. 1,3-bis(2-chloroethyl)-1-nitrosourea (NSC 409962) in the treatment of brain tumors. *Cancer Chemother Rep* 1970; **54**: 273–81.
4. Wechsler W, Kleihaus P, Matsumoto S, *et al.* Pathology of experimental neurogenic tumors chemically induced during prenatal and postnatal life. *Ann NY Acad Sci* 1969; **159**: 360–408.
5. Pinder RM, Brogden RN, Sawyer PR, *et al.* Metoclopramide: a review of its pharmacological properties and clinical use. *Drugs* 1976; **12**: 81–131.
6. Joss RA, Brnad BC, Buser KS, *et al.* The symptomatic control of cytostatic drug-induced emesis. A recent history and review. *Eur J Cancer* 1990; **26**: 52–8.
7. Lybak S, Kljellén 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.
8. Lybak S, Kjellén E, Nilsson P, *et al.* Normal tissue reactions and optimization of dose schedule for metoclopramide in combination with ionizing radiation. *Acta Oncol* 1992; in press.
9. 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.
10. 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.
11. Gralla RJ, Itri LM, Pisko SE, *et al.* Antiemetic efficacy of high-dose metoclopramide: randomized trials with placebo and prochlorperazine in patients with chemotherapy-induced nausea and vomiting. *N Engl J Med* 1981; **305**: 905–9.
12. Kris MG, Gralla RJ, Tyson LB, *et al.* Improved control of cisplatin-induced emesis with high-dose metoclopramide and with combinations of metoclopramide, dexamethasone, and diphenhydramine. *Cancer* 1985; **55**: 527–34.
13. 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.
14. Brown JM, Biaglow JE, Hall EJ, *et al.* Sensitizers and protectors to radiation and chemotherapeutic drugs. *Cancer Treat Symp* 1984; **1**: 85–101.
15. Stratford IJ. Mechanisms of hypoxic cell radiosensitization and the development of new sensitizers. *Int J Radiat Oncol Biol Phys* 1982; **8**: 391–8.

(Received 27 February 1992; accepted 16 March 1992)