Cancer Chemother Pharmacol (1986) 16: 125–128

Cancer Chemotherapy and Pharmacology

In vivo studies with the novel anticancer agent mitozolomide (NSC 353451) on Lewis lung carcinoma

Massimo Broggini[1], Eugenio Erba[1], Luciano Morasca[1], Carmel Horgan[2], and Maurizio D'Incalci[1]

[1] Mario Negri Institute for Pharmacological Research, via Eritrea 62, I-20157 Milan, Italy
[2] CRC laboratories, Department of Pharmacy, University of Aston, Birmingham B4, 7ET, United Kingdom

Summary. Mitozolomide is one of the most effective drugs against Lewis lung carcinoma in the mouse. Two IP doses of 40 mg/kg (days 6 and 15 after IM transplantation of 3LL) or four doses of 20 mg/kg given at various intervals (starting from day 6) increased survival time by 100%. A single IP dose of 80 mg/kg was toxic, and 10 mg/kg was ineffective even when this dose was given on eight occasions.

The pharmacokinetics of mitozolomide was investigated in 3LL-bearing mice by HPLC assay. Peak drug levels were achieved in tumor 15 min after IP treatment, after which they declined according to first-order kinetics, with a half-life of 80–100 min (the same as in plasma). No dose-dependent kinetics was observed.

Flow cytometry studies showed an accumulation of 3LL cells in G2M 24 h after drug treatment. This cell cycle perturbation was reversed 96 h after the inactive dose of 10 mg/kg, but not after the effective dose of 40 mg/kg.

Introduction

Mitozolomide (NSC 353451) or 8-carbamoyl-3-(2-chloroethyl)-imidazo-[5,1-*d*]-1,2,3,5-tetrazin-4(3*H*)-one (Fig. 1), is a new compound with high antitumor activity against several experimental tumors [8, 14]. It has completed phase I evaluation and is now undergoing a phase II study.

Its wide spectrum of activity, its similarity to chloroethylnitrosoureas in the formation of DNA-interstrand cross-links [6] and its reported cross-resistance with nitrosoureas [5] are consistent with a mechanism of action involving the formation of a chloroethyltriazene (MCTIC). Furthermore, in preliminary studies mitozolomide and MCTIC have shown similar cytokinetic effects on 3LL cells [10]. It has been postulated that carbamoylating activity could to some extent be responsible for the bone marrow toxicity of nitrosoureas [11]; therefore, mitozolomide, which does not form carbamoylating species [9], may offer advantages.

Studies on the antineoplastic, pharmacokinetic, and pharmacodynamic properties of this anticancer agent should provide useful information for setting up rational, safe, clinical studies.

In this paper we show evidence that the dosage schedule is crucial for the antitumor activity and toxicity of mitozolomide. In addition, tumor drug concentrations and flow cytometry studies after active or inactive drug doses are reported.

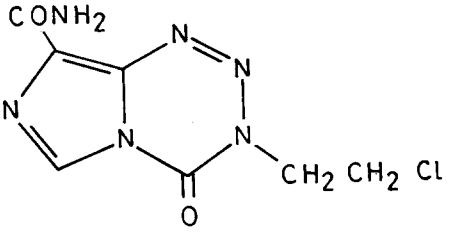

Fig. 1. Chemical structure of mitozolomide

Materials and methods

Drug. Mitozolomide was kindly supplied by Prof. M.F.G. Stevens, Aston University, Birmingham, UK.

Animals and tumor. C57Bl/6 male mice (20±2 g body weight) obtained from Charles River, Italy, were used for these experiments. Mice each received an IM transplant of 10^5 cells/0.1 ml body weight of Lewis lung carcinoma (3LL), which was maintained in the same strain of mice by IM transplant every 2 weeks.

Antitumor activity. For evaluation of antitumor activity, mice bearing IM 3LL were treated IP with mitozolomide dissolved in dimethylsulfoxide (DMSO):saline (0.5:9.5). At 21 days after tumor transplantation, a group of mice was killed and primary tumor and metastasis weights were recorded. In other experiments only survival time was recorded.

Drug concentrations in tumor. Tumor levels of mitozolomide were determined in mice bearing 15-day-old IM 3LL (average tumor weight 2.2±0.2 g) after single IP doses of 10, 20 or 40 mg/kg. At 5, 15, 30, 60, 120, 240, 360, and 1440 min after injection, four animals per point at each dose level were killed by decapitation and tumors were removed and frozen at −20 °C until use. Tumors were homogenized in 5 vol. distilled water, and 0.5 ml homogenate was used. After extraction with ethyl acetate the samples were analyzed by HPLC with spectrophotometric detection according to Slack et al. [12]. Recovery of the extraction was 85%, and sensitivity was 0.05 μg/g.

Offprint requests to: M. Broggini

Flow cytometry analysis. The tumors were removed and washed in a petri dish with PBS, after which fragments were minced with scissors to separate the vegetative part, which was used for cell analysis, from the necrotic part. Tumor fragments were forced through a 18G × 11/2 needle and resuspended in Hank's solution maintained at 4 °C. The cells were stained with propidium iodide (P.I.) (Calbiochem Behring, US) by adding 3 ml P.I. solution (50 μg/ml in 0.1% sodium citrate plus 30 μl Nonidet P40) to 200–300 μl cell suspension plus 30 μl RNAse (1 mg/ml) (Calbiochem Behring, US), which was stored at room temperature before the DNA analysis. Fresh leukocytes from C57Bl/6 mice, stained as 3LL cells, were used as standard. Flow cytometry analysis was performed using a 30L Cytofluorograph (Ortho Instruments, US). The coefficient of variation (CV) of the standard was between 1.5% and 2.5%, and that for the G0-G1 phase peak of 3LL cells was 4%–6%. At least 50 000 cells were measured; cell cycle analysis was performed using the previously described method on a HP85 computer [3]. The A/B ratio is the ratio between the diploid normal cells, A (channels 35–45), and the tetraploid tumor cells, B (channels 70–170).

Pharmacokinetic analysis. The areas under the drug concentration versus time curves (AUC) were calculated by trapezoidal integration. Elimination half-life was calculated by regression analysis.

Results

The antitumor and antimetastatic activity of mitozolomide is illustrated in Table 1. A single dose of 10 mg/kg was virtually inactive, and 80 mg/kg was toxic. Antitumor activity was observed at doses of 20 and, particularly, 40 mg/kg. Both doses had considerable antimetastatic activity, all the animals being apparently free of metastases. Giving 20 mg/kg four times at different intervals resulted in more than 90% reduction of tumor weight, with all the animals apparently free of metastases.

Table 2 shows the effect of different dosage schedules on the survival time of 3LL-bearing mice. A single IP dose of 80 mg/kg was toxic (T/C × 100 = 76), but when the same total amount was given in fractionated doses survival time was much prolonged and there were no toxic deaths.

Table 2. Effect of different treatment schedules of mitozolomide on survival time of 3LL-bearing mice

Treatment IP (mg/kg) (day)	T/C × 100[a]
80 × 1 (10)	76
40 × 2 (6, 7)	117
40 × 2 (6, 15)	204
20 × 4 (6, 7, 8, 9)	198
20 × 4 (6, 9, 12, 15)	219
20 × 4 (6, 10, 14, 18)	200
10 × 8 (6–13)	158
10 × 8 (6, 9, 12, 15, 18, 21, 24, 27)	113
20 × 5 (6, 9, 12, 15, 18)	209
20 × 6 (6, 9, 12, 15, 18, 21)	177
20 × 7 (6, 9, 12, 15, 18, 21, 24)	149
20 × 8 (6, 9, 12, 15, 18, 21, 24, 27)	128

Mitozolomide was dissolved in DMSO: saline (0.5:9.5)
3LL was transplanted IM (10^5 cells/0.1 ml/mouse)
[a] T/C, median survival time of treated mice/median survival time of control mice × 100
Median survival time of control mice was 24 days

The highest T/C × 100 values were found with two doses of 40 mg/kg with an interval of 9 days (but not when given on successive days) or four doses of 20 mg/kg, either given every day or with 3- or 4-day intervals. Activity was low after eight doses of 10 mg/kg.

When the total amount of drug administered was increased there was a progressive decrease in activity due to toxicity until a total dose of 160 mg/kg (8 doses of 20 mg/kg) was reached. Five doses of 20 mg/kg seem to be still active and not yet toxic.

Table 3 reports tumor levels of mitozolomide after a single IP dose of 10, 20, or 40 mg/kg to 3LL-bearing mice. Peak levels were obtained 15 min after injection and were 3.6, 7.2 and 14.9 μg/g, respectively. The disappearance of the drug was monoexponential, with half-lives in the range of 80–100 min. The corresponding AUC values were 473.5, 931.2 and 1623.0 μg/g × min respectively.

Figure 2 shows the DNA pattern of 3LL before (a–e) and 24, 48, 72, 96, and 168 h after a single inactive dose of 10 mg/kg (f–j) or single active dose 40 mg/kg (k–o) of mi-

Table 1. Antitumor activity of different doses of mitozolomide in 3LL-bearing mice

Dose mg/kg	Tumor weight inhibition (%)	Weight inhibition of metastases (%)	Mice free of metastases (%)
10 × 1 (14)	6	53	0
20 × 1 (14)	24	100	100
20 × 1 (10)	37	99	88
40 × 1 (14)	57	100	100
80 × 1 (10)	toxic	toxic	toxic
20 × 4 (6,9,12,15)	99	100	100
20 × 4 (6,10,14,18)	98	100	100

Mitozolomide was dissolved in DMSO: saline (0.5:9.5) and injected IP. Autopsy was performed 23 days after tumor transplantation: mean control tumor weight was 10.059 ± 0.49 g; mean weight of control metastases was 40.6 ± 7.9 mg. Figures in parentheses give the day(s) of treatment

Table 3. Distribution of mitozolomide in tumor of mice bearing IM 3LL

Dose (mg/kg) IP	Tumor peak level (μg/g)	Tumor AUC 5→360 min μg/g × min	Half-life (min)
10	3.57 (15 min) ±0.04	473.5 ±75.7	91.4
20	7.22 (15 min) ±0.56	931.2 ±141.7	106.0
40	14.92 (15 min) ±2.31	1623.0 ±185.6	83.7

Mice bearing IM 3LL (10^5 cells/0.1 ml body weight) were given the drug IP 14 days after tumor inoculm

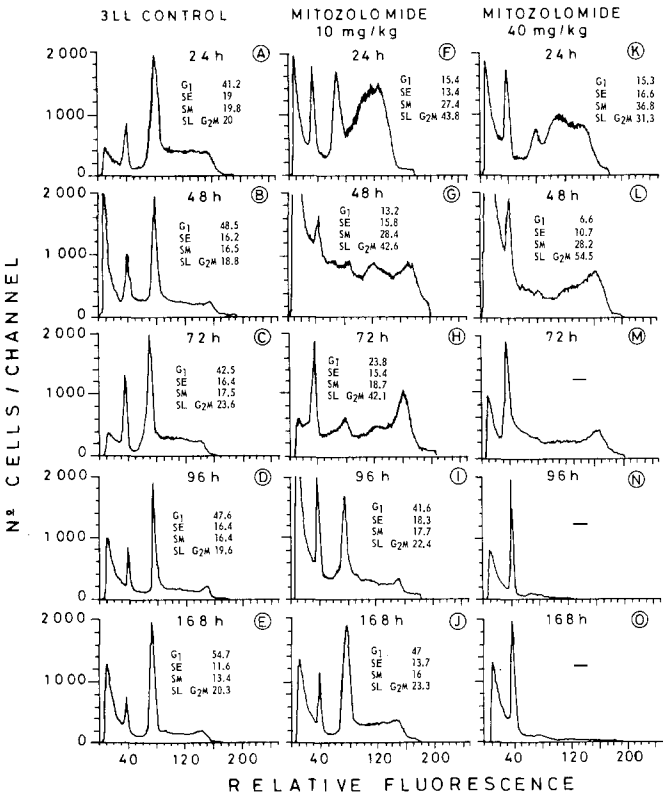

Fig. 2. Flow cytometry DNA histograms of 3LL without mitozolomide treatment (controls) and at intervals (24, 48, 72, 96 and 168 h) after mitozolomide (10 or 40 mg/kg IP)

tozolomide in mice bearing 10-day-old IM 3LL. From left to right, in each histogram the following peaks are seen: after a variable shoulder of nuclear debris, the first represents the G0-G1 peak of normal diploid cells present in the tumor overlapping with the mouse leukocytes used as standard; the second peak represents the G0-G1 peak of the tetraploid 3LL tumor cells; the third, less prominent, peak represents the tumor cells in G2M phase of the cell cycle, and between the G1 and G2M peaks are cells in S phase, which is further divided into early (SE), middle (SM), and late (SL) phase.

As shown in Fig. 2, only 24 h after 10 mg/kg mitozolomide there was an accumulation of cells in SL-G2M (43.8%, as against 20% in controls), with a proportional decrease of cells in G1 phase (15.4%, as against 41.2% for controls) (Fig. 2f).

The effect of treatment on cell cycle phase distribution was more evident after 48 h (Fig. 2g), with 13.2%, and 42.6% of cells in G1 and SL-G2M, respectively. After 72 h (Fig. 2h) the accumulation of cells in SL-G2M was still marked (42.1% as against 23.6% for the controls), but the percentage of cells in G1 phase was 23.8%, as against 42.5% for controls.

The cell cycle returned to the normal pattern, also seen in controls by 96 h (Fig. 2i) and 168 h (Fig. 2j) after treatment.

At 40 mg/kg mitozolomide caused a decrease of the percentage of cells in G1 phase of the cycle 24 h after treatment, which was similar to that following 10 mg/kg but with an accumulation of cells in SL-G2M also involving the SE and SM phases (Fig. 2k). After 48 h the block of cells in SL-G2M appeared more evident, with a corresponding decrease of cells in G1 phase. The A/B ratios of 0.5, 4.2, and 9.6, respectively, at 72, 96 and 168 h of treatment indicate that the number of cancer cells was strongly reduced compared with the normal population; therefore, as shown in Fig. 2m–o, no analysis of cell cycle phase distribution was possible.

Discussion

The present paper confirms in the 3LL model the high antitumoral potency described for mitozolomide in other experimental tumors [8]. As already observed for other drugs in this model [2], mitozolomide inhibited lung metastases more than the primary tumor.

The schedule of treatment used was crucial in determining activity: a total dose of 80 mg/kg, which was too toxic to show any activity when given as a single dose, gave a good increase in survival time without toxic deaths when split into two or four doses. However, the decrease in toxicity parallels the decrease in activity, and 10 mg/kg, which was inactive as a single dose, had no activity even when given repeatedly up to a total dose of 80 mg/kg.

Studies on the distribution of mitozolomide in Lewis lung tumors showed that, at least in the range of doses utilized, kinetics were not dose-dependent, since the AUC values were proportional to the dose and similar half-lives were found for each dose. This is in agreement with previous observations in humans, in which similar half-lives were found in plasma for doses ranging from 8 to 115 mg/m^2 [13].

The half-life of mitozolomide in 3LL tumor was similar to that in plasma (data not presented; [1]), and approximately the same as when the drug was incubated at 37 °C in plasma (data not reported) or in buffer solution at pH 7.4 [14]. This suggests that the drug undergoes spontaneous chemical decomposition and is not significantly metabolized by enzymatic systems. Since the half-life is relatively short (<2 h) no drug accumulation occurs when repeated daily doses are given to mice.

Considering that the dose of 10 mg/kg is not active, our data indicate that tumor peak levels of mitozolomide above 4 μg/g or AUC greater than 500 μg/g × min are needed to obtain antitumor activity against mouse 3LL.

Using two doses, one completely inactive in inhibiting tumor weight gain (10 mg/kg) and one which gives more than 50% inhibition (40 mg/kg), we detected interesting differences in cell cycle perturbation, which might help ex-

plain the different activity. After a first block of cells in SL-G2M phases observed with both doses, after 10 mg/kg there was a gradual return of cells in cycle, and 96 h after treatment no differences could be found between controls and treated populations.

With the higher dose the block became irreversible, with a sharp decrease in the cancer cell population in the DNA histogram.

Though no studies are available on the mechanism of repair of mitozolomide-induced DNA damage [6, 7], it is possible that the transalkylation of the chloroethyl moiety from 06-guanine to a cystine-containing protein may occur, as already described for chloroethylnitrosoureas [4]. This is a reasonable hypothesis, since both chloroethylnitrosoureas and mitozolomide, in spite of their different chemical properties, can produce the same alkylating species [9, 15] (i.e., chloroethyldiazohydroxides). It is therefore conceivable that the levels of 06-guanine-alkyltransferase in the 3LL cells are sufficiently high to remove the 06-guanine adducts formed after the inactive dose of 10 mg/kg. When higher doses are used (e.g., 40 mg/kg), 06-guanine alkyltransferase is perhaps saturated so that DNA damage persists, thus eventually leading to cell death.

On the basis of these considerations we believe that the use of flow cytometry for evaluation of repair of mitozolomide-induced DNA damage, as determined by restoration of cell cycle progression after drug treatment, could prove useful in selection of human tumors possible deficient in 06-guanine alkyltransferase (or any other enzyme playing an important role in DNA repair) and thus susceptible to the drug's action.

We are currently using this approach to test different cell lines or primary cultures derived from human tumors, and the results so far indicate that even though a large proportion of human cancer cells appear to recover efficiently from mitozolomide-induced cell cycle perturbation, there are some in which the effects appear irreversible.

Acknowledgements. The generous contribution of the Italian Association for Cancer Research, is gratefully acknowledged.

References

1. Brindley CJ, Antoniw P, Newlands ES, Bagshawe KD (1984) Plasma and tissue disposition of CCRG 81010 (NSC 353451) in mice. Proc Am Assoc Cancer Res 25: 355
2. Donelli MG, Garattini S (1977) Differential accumulation of anticancer agents in metastases compared with primary tumors in experimental models. In: Tagnon HJ, Staquet MJ (eds) Recent advances in cancer treatment. Raven, New York, p 177
3. Erba E, Ubezio P, Colombo T, Broggini M, Torti L, Vaghi M, D'Incalci M, Morasca L (1983) Flow-cytometric analysis of DNA distribution after VP16-213 treatment of Lewis lung carcinoma. Cancer Chemother Pharmacol 10: 208
4. Erickson LC, Laurent G, Sharkey NA, Kohn KW (1980) DNA cross-linking and monoadduct repair in nitrosourea treated human tumor cells. Nature 288: 727
5. Gibson NW (1982) Investigation into the mechanism of action of antineoplastic nutrosoureas. PhD Dissertation, University of Aston in Birmingham
6. Gibson NW, Erickson LC, Hickman JA (1984a) Effects of the antitumor agent 8-carbamoyl-3-(2-chloroethyl)imidazo [5,1-*d*]-1,2,3,5-tetrazin-4(3*H*)-one on the DNA of mouse L1210 cells. Cancer Res 44: 1767
7. Gibson NW, Hickman JA, Erickson LC (1984b) DNA cross-linking and cytotoxicity in normal and transformed human cells treated in vitro with 8-carbamoyl-3-(2-chloroethyl)imidazo[5,1-*d*]-1,2,3,5-tetrazin-4(3*H*)-one. Cancer Res 44: 1772
8. Hickman JA, Gibson NW, Stone R, Stevens MFG, Lavelle F, Fizames C (1982) M&B 39565: A novel heterocycle with potent antitumor activity in mice. Proceedings of the 13th International Cancer Congress, Seattle, USA p 551
9. Horgan CMT, Tisdale MJ (1984) Antitumor imidazotetrazines: IV. An investigation into the mechanism of antitumor activity of a novel and potent antitumor agent, mitozolamide (CCRG 81010, M&B 39565; NSC 353451). Biochem Pharmacol 33: 2185
10. Horgan CMT, Tisdale MJ, Erba E, D'Incalci M, Pepe S (1983) Flow cytometric analysis of DNA distribution in Lewis lung carcinoma cells after treatment with CCRG 81010 (M&B 39565). Br J Cancer 48: 139
11. Kann HE Jr (1981) Carbamoylating activity of nitrosoureas. In: Prestayko AW, Baker LH, Crooke ST, Carter SK, Schein PS (eds) Nitrosoureas: Current status and new developments. Academic, New York, p 95
12. Slack JA, Goddard C (1985) Antitumour imidazotetrazines: VII. Quantitative analysis of mitozolomide in biological fluids by high-performance liquid chromatography. J Chromatogr 337: 178
13. Slack JA, Newlands ES, Blackedge G, Goddard C, Brindley CJ, Stevens MFG (1984) Clinical pharmacokinetics of CCRG 81010 (M&B 39565, NSC 353451) Proc Am Assoc Cancer Res 25: 164
14. Stevens MFG, Hickman JA, Stone R, Gibson NW, Baig GU, Lunt E, Newton CG (1984) Antitumor imidazotetrazines: 1. Synthesis and chemistry of 8-carbamoyl-3-(2-chloroethyl) imidazo[5,1-*d*]-1,2,3,5-tetrazin-4(3*H*)-one, a novel broad-spectrum antitumor agent. J Med Chem 27: 196
15. Weinkam RJ, Lin H-S (1979) Reactions of 1,3-bis(2-chloroethyl)-1-nitrosourea and 1-(2-chloroethyl)-3-cycloexyl-1-nitrosourea in aqueous solution. J Med Chem 22: 1193

Received February 26, 1985/Accepted June 6, 1985