Activity of Mitozolomide (NSC 353451), a New Imidazotetrazine, against Xenografts from Human Melanomas, Sarcomas, and Lung and Colon Carcinomas

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ABSTRACT

The chemosensitivity of human tumor xenografts to mitozolomide, 8-carbamoyl-3-(2-chloroethyl)imidazo[5-1-d]-1,2,3,5-tetrazin-4(3H)-one, was studied in 3 different assay systems. In concentrations of 1 to 500 µg/ml, mitozolomide completely inhibited the colony-forming ability in soft agar of cell suspensions from sarcomas, melanomas, lung and colon cancers, and a mammary carcinoma. When a panel of tumors of the different histological types was tested for its sensitivity to mitozolomide in vitro, in the 6-day subrenal capsule assay in conventional mice, and, in some cases, as s.c. growing tumors in nude mice, good agreement between the different assay systems was seen. In most cases, a very pronounced antitumor effect was observed. The efficacy of mitozolomide was as good or better than that of the drugs clinically used against the tumor types tested. Tumor size measurements and histological examinations indicated that nude mice carrying a melanoma, a small cell lung cancer, and an osteosarcoma were cured of their tumors. The approach here used for evaluating the effect of a new drug on human cancers may be useful for selecting the tumor types which primarily should be studied in clinical trials. The results indicate that clinical responses to mitozolomide may be anticipated in sarcoma, melanoma, small cell lung cancer, and possibly in colon cancer.

INTRODUCTION

In a systematic study of imidazotetrazines, a novel agent, mitozolomide (previously Azolastone; NSC 353451), with potent activity against murine tumors, has been discovered by the Cancer Research Campaign Experimental Chemotherapy Group, University of Aston, Birmingham, United Kingdom (8, 9, 22). The mechanism of action of mitozolomide has not yet been elucidated, but evidence has been presented that the formation of interstrand DNA cross-links may be involved (8, 9, 12).

In view of the promising effects of mitozolomide in murine systems (11, 22), we initiated experiments to see whether mitozolomide might be active also against human cancers and, if so, to establish which of the tumor types examined were most sensitive, to aid in the planning of subsequent clinical trials.

In this investigation, we first tested the antitumor activity of mitozolomide against cells from different xenografted cancers in a HTCF assay in vitro. When a pronounced inhibition of colony formation was observed, we next examined in the HTCF assay the efficiency of the drug on a panel of tumors for each histological type. Since in vitro test systems have inherent limitations (23), we also measured the in vivo effect of mitozolomide on the same tumors, using the 6-day subrenal capsule assay in immunocompetent mice (1–3), as well as s.c. growing tumors in athymic, nude mice (5–7, 10, 16, 18). In all 3 assay systems, marked antitumor activity against human melanomas, sarcomas, lung cancers, and colon cancers was observed.

MATERIALS AND METHODS

Animals

Male C57BL/6 × DBA/2 F1 (hereafter called B6D2F1) mice, 6 to 12 weeks old, were used for the subrenal capsule assay. BALB/c athymic, nude mice were purchased from the Laboratory Breeding and Research Center, Gl, Bomholtgaard, Ry, Denmark. NMRI nude mice were bred in our own nude mouse facility. The nude mice were housed in laminar-airflow rooms at constant temperature (24–26 °C) and humidity (30 to 50%). The cages and bedding for the nude mice were sterilized by autoclaving, and the food by γ-irradiation.

Tumors

Altogether, 18 human tumor lines were used, all of which were maintained as s.c. growing xenografts in athymic, nude mice (NMRI or BALB/c strain). Two lung cancer lines (H-249 and N-417) were kindly provided by Desmond N. Carney, National Cancer Institute-Navy Medical Oncology Branch, Bethesda, MD. One lung adenocarcinoma, T 402, was obtained from Nathan O. Kaplan, University of California, San Diego, CA. Of the 2 colon cancers, Co-115 was obtained from B. Sordat, Epalinges/Lausanne, Switzerland, and one, WiDr, as well as the mammary cancer line MDA-MB 231, were obtained from American Type Culture Collection, Rockville, MD. The other tumors (5 melanoma, 2 osteosarcoma, 3 soft tissue sarcoma, and 2 lung cancer lines) were established in this laboratory from tumor biopsies of patients at the Norwegian Radium Hospital.

Drugs

Mitozolomide (Chart 1) was a gift from M. G. Stevens, Cancer Research Campaign, Experimental Chemotherapy Group, Department of Pharmacy, University of Aston in Birmingham, United Kingdom. Abrin was prepared in our laboratory (15). The other drugs were commercial preparations obtained from the Department of Pharmacy, The Norwegian Radium Hospital.

Mitozolomide was dissolved in a minimal amount of dimethyl sulfoxide and kept refrigerated in glass tubes. Immediately before use, the drug was diluted in 0.9% NaCl solution (saline) to appropriate concentrations and injected into the animals in volumes of 0.2 ml. CCNU was dissolved...
in Cremophor EL (Sigma) and injected i.v. into B6D2F, mice and i.p. into nude mice, whereas the other drugs were used as aqueous solutions and given i.v. in volumes of 0.2 to 0.4 ml.

Assay of Anticancer Activity

**HTCF Assay.** Human tumors serially transplanted in athymic, nude mice were mechanically disaggregated by the use of a Stomacher 80 Lab-Blender (Seward Surgical, London, United Kingdom). For the sarcomas, an enzymatic procedure (digestion with collagenase, 1 mg/ml, and 1000 IU hyaluronidase, 37 °C for 1 h) was included before the mechanical disaggregation. The cell suspensions were filtered through a 4- to 5-μm nylon mesh, centrifuged, and resuspended in serum-containing Ham's F-10 medium. The cultivation in soft agar and the chemosensitivity testing were performed by a modification of the procedure of Courtenay and Mills (4) as described previously (23). The cells were incubated for 1 h with 4 different drug concentrations. Triplicate cultures were prepared in culture tubes containing rat RBC, and the tubes were incubated for 1 to 3 weeks. Colonies of more than 30 cells were scored by means of a Zeiss stereo microscope. The plating efficiency was defined as the number of colonies formed as a percentage of the number of viable cells plated.

Four different concentrations of each compound, over a 3-log range, were used. Since the 50% lethal dose value of mitozolomide after a single i.v. dose was found to be 80 mg/kg, i.e., 5 times that of ADM, we used the in vitro experiments mitozolomide concentrations that were 5 times the ADM concentrations previously found to give a convenient number of colonies. The colony formation in the treatment cultures was expressed as a percentage of colony formation in the untreated control cultures.

**SRC Assay.** The assay was carried out as described previously (1, 2). Tumors grown s.c. (6 to 15 mm) were removed aseptically from nude mice and immediately placed in RPMI medium at room temperature. Viable tissue was dissected out and cut by scalpels into approximately 1-cu mm cubes which were implanted subcutaneously. The tumor size was measured by means of a stereoscopic microscope, calibrated in ocular units (OMU) (10 OMU = 1 mm).

The animals were divided into groups of 5 to 7 animals each, and in the treatment groups the mice were given the different drugs i.v. on Days 1 and 2. The doses, which are given in the legends to charts, were based on toxicity studies using the same schedule. The maximum doses giving no toxic deaths and a weight loss less than 15% were used. For mitozolomide, this dose was 40 mg/kg/dose.

Six days after implantation of the tumor tissue, the animals were sacrificed, the size of the subrenal tumor was assessed in situ by measuring 2 perpendicular diameters, and the mean tumor diameter different for each group was calculated. The antitumor activity is expressed according to the following:

\[
\text{Antitumor activity} = \frac{D_2 - D_0}{D_2}
\]

where \(D_0\) and \(D_2\) represent the mean tumor diameter in the control and treated groups, respectively.

**Xenografts in Athymic Mice.** The nude mouse experiments were carried out as described previously (5, 6). Mice with progressively growing s.c. xenografts were divided into groups of 8 to 10 animals each. When the tumors had reached a mean diameter of 6 to 8 mm, the animals were treated with the drugs in the doses indicated in the legends to charts. The maximum tolerable dose of mitozolomide, given i.v. 3 to 4 times on a once-weekly schedule, was found to be 40 mg/kg/dose.

The tumor size was assessed by measuring twice weekly 2 perpendicular diameters. By subtracting, for each diameter, the value at the start of treatment, the mean tumor diameter differences (±SE), were calculated (6). The results are presented as growth curves where the mean tumor diameter difference for each treated and untreated group is plotted versus time. At the start of treatment this value is by definition zero.

The effect of treatment was expressed numerically as the FVR (21), defined by the expression:

\[
\text{FVR} = \log_{10}\left(\frac{\text{mean } V_c}{\text{mean } V_t}\right)
\]

where \(V_c\) and \(V_t\) are the relative tumor volumes in the control group and the treated group, respectively, at a given time (usually the time when the tumor diameter difference between control and treated tumors was maximal). FVR gives the number of decades by which the treated tumor volume is reduced below the control value. Thus, e.g., a FVR value of 1.0 implies that the mean volume of the treated tumors is reduced to one-tenth of that of the control tumors (21). The tumor volume was calculated according to the formula:

\[
\frac{x}{6} (MD)^2
\]

where \(MD\) is the mean tumor diameter (6).

**RESULTS**

**Initial Screening.** The first examination of the sensitivity of different xenografts to mitozolomide involved testing in soft agar. In Chart 2A, typical results are presented for a SCLC, and in Chart 2B for a soft tissue sarcoma. In both cases, mitozolomide in concentrations of 10 μg/ml was able to completely inhibit colony formation. In the case of the SCLC cells, the dose-
response curves showed that mitozolomide inhibited colony growth in lower concentrations than ADM, CDDP, and etoposide (VP-16), drugs commonly used in the treatment of lung cancers (14). Another new drug, N-methylformamide (NSC 3051) did not inhibit colony formation of the SCLC cells in the dose range tested (5 μg to 5 mg/ml). The effect of mitozolomide on the sarcoma seemed to be comparable to those of CDDP and ifosfamide (Chart 2B). In similar experiments with a malignant melanoma, as well as colon and breast carcinomas, substantial inhibition of colony formation by mitozolomide was found.

The above data are clearly only suggestive. In vitro data can only be used to predict the drug response in vivo if they have been carefully calibrated against in vivo results, as we have emphasized previously (23). However, the above results were found sufficiently encouraging to warrant further exploration in in vivo models.

Malignant Melanomas. When a panel of melanomas was studied in the soft agar assay, it was found (Chart 3A) that the 5 melanomas tested varied greatly in their sensitivity to mitozolomide. The drug could completely inhibit the in vitro growth of all the melanomas tested, and in one case, the drug concentration required to inhibit colony formation to 50% of the control was as low as 0.5 μg/ml. The wide range of sensitivities, over several logs, suggests that the tumors tested may constitute a representative panel of human melanomas.

The effect of mitozolomide on the same melanomas was then studied in the SRC assay and compared with the effects of 2 drugs commonly used in the clinic. In some cases, the sensitivity of the tumors was tested also in the athymic, nude mouse model. The results for the melanoma LOX show that the growth of the subrenal grafts (Chart 4, left) was clearly inhibited by CDDP, slightly more by CCNU, and that the strongest effect was obtained with mitozolomide. When LOX was tested as a s.c. tumor in nude mice (Chart 4, right), mitozolomide and CCNU were clearly more active than CDDP, with fractional volume reduction values (on Day 31) of 1.95, 1.74, and 0.96, respectively. Although the relative efficacy of the 3 drugs was somewhat different in the 2 in vivo systems (Chart 4), the ranking of the drugs with respect to activity was the same.

In the experiment shown in Chart 4 (right), the control mice had to be killed on Day 31 after implantation because of the size of the tumors (>25 mm) and the poor general condition of the animals. In contrast, in the mitozolomide-treated animals the
tumors eventually disappeared and did not reappear during the observation period (52 days).

After 2 to 3 doses of mitozolomide, the grafts assumed a yellow tinge, and the tumors flattened considerably. This implies that the difference in tumor volume between the mitozolomide-treated and the other groups was in fact larger than what was inferred from the growth curves, which are based on measurements of 2 perpendicular diameters. Thus, the results indicated that in the case of mitozolomide treatment, tumor diameter measurements and FVR calculations may underestimate the effect.

The strong growth-inhibiting effect of mitozolomide on melanomas is illustrated in a representative picture, taken on Day 31 after implantation (Fig. 1) of a mitozolomide-treated and an untreated tumor-bearing athymic mouse. The diameters of the respective tumors were 5 to 7 mm and 25 to 30 mm. In sections of the s.c. tumor of a mitozolomide-treated nude mouse, no viable tumor cells were found (Fig. 3b). These had all been replaced by necrotic and fibrotic tissue. Histological sections of the corresponding subrenal capsule xenografts in immunocompetent mice, taken 4 days after treatment, showed large, swollen tumor cells with pyknotic nuclei after mitozolomide treatment (Fig. 2b), whereas in the untreated animals the subrenal graft (Fig. 2a) was morphologically similar to the s.c. graft from an untreated nude mouse (Fig. 3a). The results suggest that in this instance tumor regrowth probably would not have occurred.

Altogether, the results indicate that mitozolomide may be at least as active against malignant melanomas as CCNU, a drug that is being used in the treatment of this disease in the clinic.

Lung Cancers. In Chart 3B, the ability of mitozolomide to inhibit growth of clonogenic cells of a panel of lung cancers is shown. As was the case with the malignant melanomas, the various tumors differed widely in response to the drug, with the 2 adenocarcinomas and one SCLC tumor (BVX) being the least sensitive. This particular xenografted SCLC line was established from a patient who was resistant to a combination regimen that included ADM.

The response of the lung cancer H-249, growing s.c. in athymic mice, is shown in Chart 5. The tumors of the mitozolomide-treated mice disappeared almost completely (FVR $\geq 2.78$). In contrast, ADM, CDDP, and etoposide (VP-16) had only minor effects on tumor growth (FVR 0.19, 0.22, and 0.07, respectively). However, linear regression analysis, performed on the individual values at each time point for each group, demonstrated that also these 3 drugs had statistically significant growth-inhibiting effects ($P \leq 0.003$).

Sarcomas. In Chart 6 (left), the effect of mitozolomide on different sarcomas in vitro is demonstrated. Comparison with Chart 2 shows that the individual sarcomas differed to a lesser extent in their sensitivity to mitozolomide than the melanomas and the lung cancers.

In the SRC assay (Chart 7, left), mitozolomide had a much greater growth-inhibiting effect on the osteosarcoma TPX than ADM and the protein synthesis inhibitor, abrin (17), which seemed to be equally effective. The concomitant studies in nude mice (Chart 7, right) showed that, after 2 doses of mitozolomide, the s.c. grafts started to decrease in size, and at Day 48 the FVR value was >0.80. In some of the animals, the tumors finally disappeared completely without recurring, whereas in the other mice of the group, the tumors were still decreasing slowly when the experiment had to be terminated as the animals started to die, for reasons unrelated to tumor growth or drug toxicity. Abrin had a statistically significant ($P = 0.02$) growth-inhibiting effect (FVR = 0.09), whereas ADM had no effect (FVR = 0).

Like the malignant melanoma grafts, the sarcoma grafts assumed a yellow tinge when the mice had received 2 to 3
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Mitosolomide concentration (µg/ml)

Chart 6. Dose-response curves in vitro of tumor cells prepared from 5 different sarcoma xenografts (A), 2 colon cancer xenografts, and one mammary cancer xenograft (B), treated with increasing concentrations of mitozolomide for 1 h at 37 °C. The sarcomas included 2 osteosarcomas, OHX (A) and TPX (O); one leiomyosarcoma, MTMX (O), one hemangiopericytoma, DEEX (O); and one malignant schwannoma (x). The 2 colon cancers were Co-115 (x) and WiDr (O), and the mammary cancer was MDA-MB-231 (C).

DISCUSSION

The results presented in this paper demonstrate that mitozolomide, a new imidazotetrazine, with broad-spectrum anticancer activity in murine tumors (11, 22), was highly active against several xenografted human tumors, as measured in 3 different assay systems. Of particular significance is the finding that of the 6 different human tumor lines so far tested as tumors growing s.c. in immunodeficient mice, a system generally assumed to reflect the sensitivity of the parent tumors (5, 7, 10, 13, 16, 18, 20, 21), 5 showed a strong response to mitozolomide.

It is well known that, in tumor-bearing animals, procedures causing a strong weight loss may in themselves induce tumor growth retardation. In our study, control experiments showed that weight losses of the magnitude found in the drug-treated animals did not affect tumor growth.

The effect of mitozolomide on the responsive tumors appeared early. Thus, after 2 to 3 courses of mitozolomide treatment, a yellowish appearance and flattening of the treated tumors was evident, and histological examination revealed that already at this stage the tumor cells were dead or dying. Three of the lines (a melanoma, a SCLC, and a sarcoma), eventually disappeared completely upon mitozolomide treatment, and none of these reappeared during the observation period of more than 60 days. These findings, together with the absence of tumor cells by histological examination, suggest that the treated mice may have been cured. For comparison, 3 of the drugs most commonly used in the clinic against SCLC gave only minor growth inhibition of the SCLC tumor tested here. Also, in the case of the sarcoma, the other drugs tested were far less active than mitozolomide in the doses tested.

In this study, the antitumor activity in vivo of the different drugs was compared at the highest doses not giving toxic deaths or unacceptable weight loss. Dose-effect curves were determined in the case of the 6-day SRC assay (1), but not in nude mouse experiments. However, since equitoxic doses were used, under conditions similar to those used in the clinic, our data presumably reflect the relative antitumor activity of the drugs tested.

Activity of mitozolomide against human tumors has not been demonstrated previously, with the exception of one human lung cancer line (LX-1; National Cancer Institute screen), found to be sensitive, when tested as a subrenal xenograft (22). The pronounced activity consistently observed here against several human tumor forms for which there is currently no satisfactory chemotherapy, is obviously of considerable clinical interest.

Mitosolomide is chemically similar to the nitrosoureas. Like these, it probably acts by causing DNA interstrand cross-link formation (8, 9, 12). In our human melanomas, it showed a similar pattern of antitumor activity as CCNU. In a Phase I trial, mitozolomide caused thrombocytopenia,4 similar to what is typical for CCNU. In the light of these similarities, the spectrum of activity in human tumors of these 2 drugs should be compared. Such studies have been initiated in our laboratory.

In spite of large efforts, rather few new, clinically effective anticancer drugs have become available in recent years. Numerous drugs are being produced and screened in murine test systems (24), but many drugs that show promise in experimental systems prove to be ineffectual in patients. Conversely, screen-

4M. F. G. Stevens, personal communication.
Chart 8. Chemosensitivity profile for the colon cancer xenograft WiDr in the 6-day SRC assay in immunocompetent mice (left) and in the s.c. nude mouse assay (right). The mice were divided into groups of 6 to 8 animals, and in the treatment groups the drugs were administered i.v. at the indicated times (arrows). The drug doses used in the SRC assay were: 5-fluorouracil, 80 mg; mitozolomide, 40 mg; and abrin, 250 ng/kg/dose. In the s.c. assay, the doses were: 5-fluorouracil, 100 mg; mitozolomide, 30 mg; and abrin, 350 ng/kg/dose. The tumors were measured twice weekly, and the mean tumor diameter difference was calculated.

ing of new compounds on human tumor cells may possibly detect anticancer activity that would not be discovered in murine tumor models (19).

The strategy here used for preclinical evaluation of new anticancer drugs may be of some general interest. By this procedure, tumor types with high probability of clinical response to the drug may be selected. The effect of mitozolomide on human cancer cells was first demonstrated in a soft agar assay, used for preliminary screening. The results suggested that mitozolomide may be at least as effective as several established agents against the tumors studied. However, since experiments on single cell suspensions do not reflect the importance of factors such as the pharmacokinetic properties of the drugs, their ability to penetrate into the tumor, and their toxicity to normal tissues, in vivo experiments are necessary before definite conclusions can be drawn, and in our opinion promising agents should be evaluated in human tumor models in vivo before being introduced in the clinic. We therefore measured the effect of mitozolomide in vivo on the same tumors transplanted under the renal capsule of immunocompetent mice as well as when they were growing s.c. in athymic, nude mice.

Since the validity of the nude mouse model is well established (5, 7, 10, 13, 16, 18, 20, 21), it may be used as a reference system. It is of interest from a methodological point of view that the results obtained with the 2 in vivo systems were by and large in good agreement. Thus, when the effects of different drugs were compared, almost without exception, the same ranking order was obtained with the 2 models. The results provide strong evidence that the 6-day SRC assay in immunocompetent mice, carried out on xenografted human tumors, can be used in evaluation of anticancer activity of new drugs. Moreover, good correlation between the in vitro and in vivo chemosensitivity data was also seen. Thus, the pronounced responses in the nude mouse and SRC models were observed in tumors that were highly sensitive in the HTCF assay. It should be noted that some of the tumors not yet tested in vivo (melanomas, sarcomas, lung cancers, and a colon cancer) appeared to have high in vitro sensitivities (Charts 3 and 6), and may be expected to respond to mitozolomide in vivo.

The encouraging results in this study should expedite clinical evaluation of the antitumor activity of mitozolomide. They suggest that sarcomas, melanomas, and possibly colon cancers should be studied in Phase II trials of mitozolomide. Among the lung cancers, SCLC tumors seem to be the most promising candidates. If the tumor panels here tested are indeed representative for their histological types, significant clinical responses may be anticipated.

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Fig. 2. Effect of mitozolomide treatment on the histology of a malignant melanoma xenograft (LOX) growing under the renal capsule of conventional mice. Untreated controls (a) and after mitozolomide treatment (b). × 620.
Fig. 3. Effect of mitozolomide treatment on the histology of a malignant melanoma xenograft (LOX) growing s.c. in athymic nude mice. a. Untreated controls, ×620. Inset, ×620. b. After mitozolomide treatment, ×155. Inset, ×620.
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