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In Vitro and *in Vivo* Inhibition of Glioblastoma and Neuroblastoma with MDL101731, a Novel Ribonucleoside Diphosphate Reductase Inhibitor

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ABSTRACT

We examined the effects of MDL101731, a novel ribonucleoside reductase inhibitor, against human glioblastomas and neuroblastoma, both *in vitro* and in xenograft models, to determine its activity against malignant brain tumors. MDL101731 produced a concentration-dependent inhibition of both glioblastoma cell lines (HS683 and J889H) and neuroblastoma (SK-N-MC) in nanomolar concentrations (IC_{50}, 30–90 nM). s.c. xenografts of human glioblastoma (D54) in athymic mice increased to five times their initial volume at a median of 7.4 days in control animals, while tumor regression occurred in 12 of 12 animals treated with MDL101731 (100 mg/kg, i.p., two times/week) during 22 days of treatment ($P < 0.0001$). Intracerebral implants of D54 carried a median survival of 20 days in control animals, whereas animals receiving MDL101731 (100 mg/kg, i.p., two times/week, days 10–35) had a median survival of 46.5 days ($P < 0.0001$). Intracerebral xenografts of SK-N-MC in athymic mice resulted in a median survival of 23 days in control animals and 26 days in animals treated with carmustine (1,3-bis(2-chloroethyl)-1-nitrosourea 20 mg/kg/week, i.v. × 2; difference not significant). There was 90% survival in animals treated with MDL101731 (200 mg/kg, i.v., two times/week, days 7–35) up to 90 days after implant. These studies indicate that MDL101731 has potent antiproliferative activity against human malignant brain tumors.

INTRODUCTION

Over the past 2 decades, very little progress has been achieved in identifying chemotherapeutic agents that significantly improve the length of survival for patients with malignant gliomas. Specifically, no single agent or combination of agents has been shown to be more effective than nitrosoureas, which at best provide a modest increase in the number of patients that survive beyond one year (1–3). Malignant gliomas continue to present a difficult therapeutic challenge and new, more effective forms of treatment are needed.

This report demonstrates the results of *in vitro* assays and xenografts of human malignant brain tumors treated with MDL101731, a novel compound synthesized to exert irreversible and potent inhibition of ribonucleoside reductase (4–6). Ribonucleoside reductase, an enzyme that catalyzes a rate-limiting step in DNA synthesis, has increased activity in rapidly growing tumors and serves as a potential target for chemotherapy (7). Prior work with conventional agents that are inhibitors of ribonucleoside reductase have been shown to be of moderate benefit in the treatment of selected human solid tumors (8–9). MDL101731 was synthesized at the Marion Merrell Dow Research Institute (Cincinnati, OH) and has shown significant *in vitro* activity against a variety of human tumor cell lines (IC_{50}, 5 to 100 nM) as well as dramatic tumor regressions in xenografts of human breast, colon, and prostate tumors in nude mice (4–6). Our studies extended the range of activity of MDL101731 to include human malignant gliomas and neuroblastoma.

MATERIALS AND METHODS

Tumors. Human glioblastoma HS683 was obtained from American Type Culture Collection (Rockville, MD). Human glioblastoma J889H is a cell line developed in the Neuro-Oncology Laboratory at Yale University (10). These cell lines were maintained in DMEM with 10% FCS, penicillin (100 μg/ml), and streptomycin (100 μg/ml) in an atmosphere of 5% CO_2 at 37°C. Subconfluent cultures were passed (1:10) by lifting the cells with 0.25% trypsin in 5 mM EDTA. At the time of testing, cells were detached with trypsin, counted on a hemocytometer, and plated at the appropriate density.

D54 is a subline of the established human glioblastoma line A172 (11–12) maintained by serial transplantation in athymic mice. It was provided by Dr. Darell Bigner (Duke University, Durham, NC). The experiments described for xenograft implants of D54 were in the fourteenth animal passage level.

SK-N-MC neuroblastoma cells were obtained from American Type Culture Collection and cultured in Eagle's modified essential medium with 10% FCS, nonessential amino acids (0.1 mM), and sodium pyruvate (0.1 mM). Different cell lines were used for the *in vitro* and *in vivo* experiments because of restricted access of D54, and HS683 and J889H do not grow as xenografts.

Clonogenic Assay. *In vitro* chemosensitivity testing was performed on human glioblastoma cell lines HS683 and J889H as well as human neuroblastoma cell line SK-N-MC. Clonogenic survival assays were performed according to the method of Tishler *et al.* (13) and Yoshida *et al.* (14). Cells were plated (10^3/well) in 6-well plastic plates and allowed to attach overnight. Wells were examined to ensure single-cell suspensions. The treatment protocol was designed so that each drug concentration was represented by at least six wells. Cells were incubated in active drug (0 to 1000 nM) for 24 h, at which time the drug was removed and replaced with fresh medium. Cultures were maintained for 7 to 14 days, and the colonies (>50 cells) were counted (×8 lens) after the wells were washed with PBS and stained with cresyl violet (0.25%) in methanol. The clonogenic capacity from treatment groups was expressed as a percentage of control wells. The concentration of drug that reduced clonogenicity by 50% (IC_{50}) compared with control was calculated.

s.c. and Intracerebral Xenografts of D54. For s.c. xenograft experiments, tumors were removed from donor animals under sterile conditions, passed through a series of screens and needles of decreasing bore, and 0.05 ml of the tumor cell suspensions was implanted into the flanks of 24 mice. The tumors were serially measured in two dimensions with calipers. When the median tumor size reached 200 mm^3 (day 0 for survival curve) animals were divided into two groups of 12 (control group: average 201, median 205, range 131–326; treatment group: average 210, median 202, range 131–332) so that there was no significant difference in average tumor size between groups. One group received MDL101731 (100 mg/kg i.p. two times/week from days 0 to 22) and control animals received and equal volume of vehicle (saline) on the same schedule. Serial measurements were continued until the tumors exceeded five times their original volume. Efficacy of treatment was quantified by the difference between treatment and control groups in median time for the tumors to reach five times their initial treatment volume. Statistical analysis was done using the Wilcoxon rank-sum test using the number of days to five times treatment size for each tumor. In addition, the number of tumors regressing (*e.g.*, becoming smaller than the initial treatment size at any time after treatment) was compared between the two groups using the Fisher's exact test.

For the intracranial tumor experiments, the same procedure was used to obtain tumors for implantation. Implantation (5 μl) at a depth of 4 mm into the right cerebral hemisphere of 25 anesthetized animals was performed on day 0.

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Table 1 *Concentration of MDL101731 that produces 50% inhibition of clonogenic capacity in human glioblastoma (HS683 and J889H) and neuroblastoma (SK-N-MC)*

Cell line	IC_{50} (nM)
J889H	30
HS683	50
SK-N-MC	90

Animals were weighed twice weekly until death. On day 10, the animals were divided into two groups. One group of 12 animals received MDL101731 (100 mg/kg i.p. two times/week for eight treatments, days 10 to 35), and the 13 control animals received an equal volume of vehicle (saline) on the same schedule. The Wilcoxon rank-sum test was used to compare survival time from tumor implantation between the two groups.

Intracerebral Xenografts of SK-N-MC. Intracerebral tumors were inoculated into male athymic nude mice (Harlan Sprague-Dawley, Inc. Indianapolis, IN) according to a similar protocol as described above (15). BCNU[2] (carmustine; Bristol Laboratories, Evansville, IN) was administered (20 mg/kg i.v.) in 5% dextrose solution containing 10% ethanol once a week. MDL101731 (200 mg/kg i.v.) was administered in saline twice each week (Tuesday and Friday). Control animals received vehicle according to the same schedule. Ten animals were used for each treatment (total 30). Treatment began 7 days after tumor implant, and the experiment was terminated on day 93 after tumor inoculation.

RESULTS

***In Vitro* Inhibition of Glioblastoma and Neuroblastoma.** MDL101731 produced a concentration-dependent inhibition of clonogenic capacity over the dose range of 0 to 1000 nM in all three cell lines (HS683, J889H, and SK-N-MC). Table 1 demonstrates similar inhibition (IC_{50}, 30 to 90 nM) for three cell lines.

D54 s.c. Xenografts. Treatment with MDL101731 or vehicle began when tumors reached 200 mm^3 (day 0) and continued until day 24 (Fig. 1). Median time from treatment to five times tumor size was 7.4 days (6.1 to 9.9 days) in the control group and 44.0 days (32.0 to 48.7 days) in animals receiving MDL101731 (median survival treatment − median survival control = 36.6 days). There were 12 of 12 tumor regressions in the treatment group compared with 0 of 12 in the control animals. Both these observations are significant ($P < 0.0001$). There was no treatment-related mortality. Average weight loss in the animals receiving MDL101731 was 0.2% of their initial body weight (0 to 4.6%). None of the control animals lost weight.

D54 Intracerebral Xenografts. Treatment with MDL101731 or vehicle began on day 10 until day 35. Median survival from implantation in the control animals was 20.0 days (17 to 25 days). Median survival in the group receiving MDL101731 was 46.5 days (29 to 49 days; (Fig. 2). This represents a 233% increase in life span and was significant at $P < 0.0001$.

Xenografts of Neuroblastoma. Drug therapy was started on day 7 after implant. BCNU was discontinued after day 21 because of progressive tumor-related morbidity. MDL101731 was administered until day 35, and animals were monitored for an additional 55 days. Intracerebral xenografts of SK-N-MC were lethal in 100% of control animals by day 31. Animals receiving MDL101731 demonstrated a 90% survival at day 90. This compares favorably with animals treated with BCNU who had a 90% mortality from tumor on day 45 (Fig. 3). No significant treatment-related toxicity was noted.

DISCUSSION

The results of these studies provide strong experimental evidence that MDL101731 may be an effective agent in the treatment of human malignant brain tumors. The *in vitro* studies demonstrating an IC_{50} in nanomolar concentrations indicate that MDL101731 has potent antiproliferative activity against both glioblastoma and neuroblastoma cell lines. More importantly, the xenograft studies with human ma-

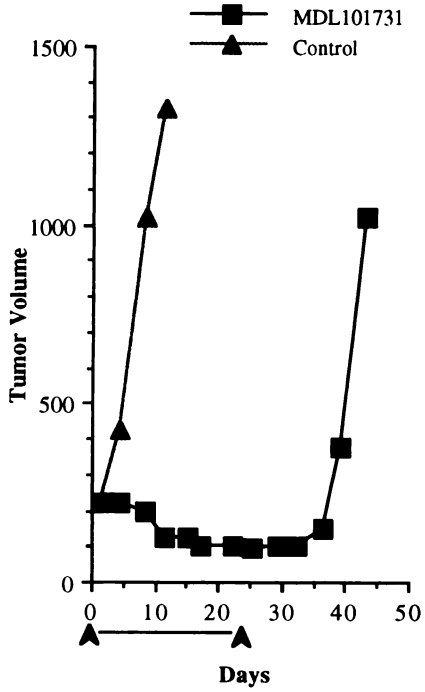

Fig. 1. Inhibition of s.c. xenografts of D54 in nude mice by MDL101731 compared with control. Points represent median tumor volumes. Tumor regression by 75% of the original treatment volume occurred in animals receiving MDL101731 (100 mg/kg × 2/week i.p.). *Arrowheads*, treatment period. Tumor growth returned after the end of treatment.

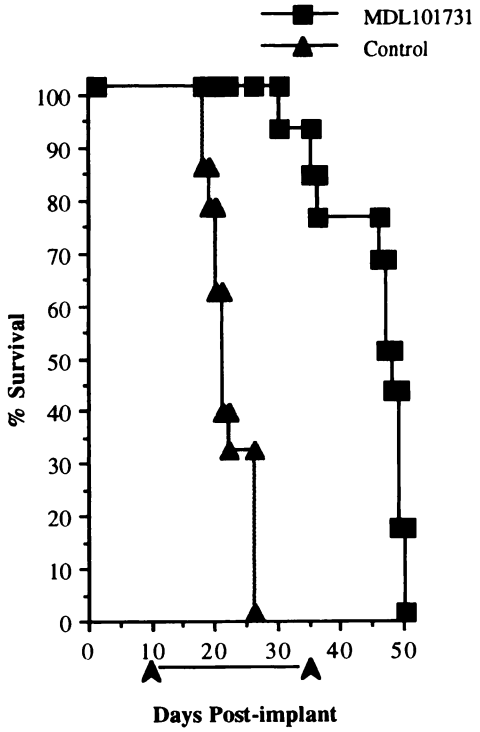

Fig. 2. Inhibition of D54 intracerebral xenografts nude mice by MDL101731 compared with control. All control animals died from tumor between days 17 and 25, while animals receiving active drug had a 75% survival by day 42. MDL101731 was administered (100 mg/kg × 2/week i.p.) between days 10 and 35. *Arrowheads*, treatment period.

[2] The abbreviation used is: BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea.

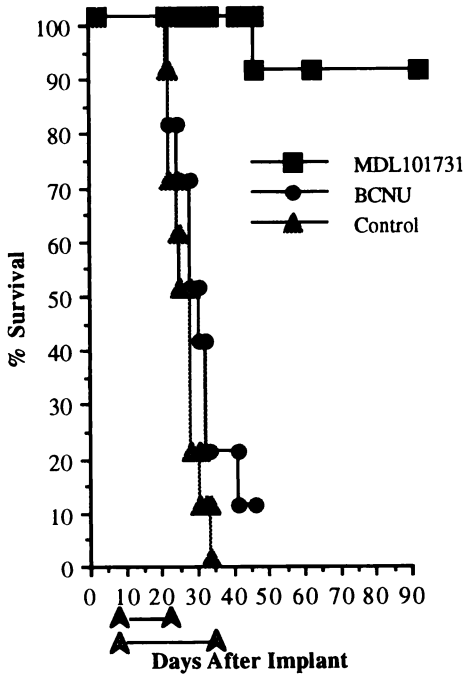

Fig. 3. Inhibition of SK-N-MC intracerebral xenografts in nude mice by MDL101731 or BCNU compared with control. All control animals died from tumor between days 15 and 31. BCNU was stopped at day 21 because of progressive tumor-related morbidity, with a 90% mortality by day 45. MDL101731 resulted in 90% survival up to day 90. *Closed arrowhead,* BCNU treatment. *Open arrowhead,* MDL101731 treatment.

lignant gliomas showing regression of s.c. tumors and dramatic prolongation of survival in intracerebral implants support the potential usefulness of this agent. Toxicology studies[3] in mice, dogs, and ferrets at the doses used in these experiments indicate that MDL101731 is associated with mild reversible myelosuppression. Formal toxicology assessments were not performed as part of our experiments; however, significant treatment-related toxicity (behavioral changes, weight loss, and poor eating) was not observed. Inhibition of s.c. and intracerebral xenografts could be achieved at a relatively low dose (100 mg/kg). Progressive tumor growth following the end of treatment indicate that this regimen is not curative. A more prolonged response was achieved at the higher dose (200 mg/kg) with no significant increase in toxicity. Survival extended up to 55 days after treatment was terminated. The response of intracerebral and s.c. malignant gliomas to MDL101731 compare favorably with response rates of other conventional drugs including alkylating agents (15). The results of the current study correlate well with prior *in vitro* and xenograft studies of MDL101731 against malignant breast, colon, and prostate tumors, which demonstrate significant tumor regression in tumor implants as well as a reduction in the number of metastases (4–6).

The ability of an agent to gain access to the tumor in the brain in cytotoxic concentrations appears to be a major limitation for systemic therapy for malignant gliomas (16). In addition, many agents have been shown to have *in vitro* activity against malignant gliomas but fail to achieve tumor control in laboratory animals (16–18). Therefore, it is important to demonstrate an agent's effectiveness in intracerebral implants. Because no direct measurements of drug concentrations were obtained in brain tumor implants, we cannot quantify the ability of MDL101731 to enter these tissues in cytotoxic concentrations; however, our results indicate that MDL101731 can significantly prolong survival of experimental animals with intracerebral xenografts.

Since direct measurements of ribonucleoside reductase were not performed in our studies, the precise mechanism(s) of MDL101731 activity in these experiments only can be inferred from prior testing. These studies reveal that MDL101731 is a potent irreversible inhibitor through covalent binding with isolated ribonucleoside reductase and in human tumor cells retrieved from treated mice (5, 6). It is presumed that MDL101731 has similar effects on malignant brain tumors. Regardless of the precise mechanism, the above studies provide compelling evidence to suggest that MDL101731 may be an effective agent for the treatment of brain tumors.

[3] Unpublished data.

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