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Preclinical study

The relationship between the antitumor activity and the ribonucleotide reductase inhibitory activity of (*E*)-2′-deoxy-2′-(fluoromethylene) cytidine, MDL 101,731

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(*E*)-2′-Deoxy-2′-(fluoromethylene) cytidine (MDL 101,731) is a new deoxycytidine analog which shows potent antitumor activity against several human tumor models. We previously showed that MDL 101,731 inhibited human ribonucleotide reductase (RNR) in HeLa S3 human cervical carcinoma cells. Recently, it has been reported that another deoxycytidine analog, 2′-deoxy-2′-methylidenecytidine (DMDC) which also inhibits RNR from *Escherichia coli*, does not inhibit RNR in intact L1210 murine leukemia cells. MDL 101,731 was designed as an inhibitor of RNR, so it is important to know the contribution of the RNR inhibitory activity of the drug on its antitumor efficacy *in vivo*. Therefore, we examined the relationship between the antitumor activity and RNR inhibitory activity of MDL 101,731 using LX-1 human lung carcinoma which was highly sensitive to this drug. MDL 101,731 showed strong inhibition of RNR activity in LX-1 lung carcinoma by both i.v. and p.o. administration. Administration of 15 mg/kg i.v. and 30 mg/kg p.o. of MDL 101,731, doses which showed almost the same degree of antitumor activity against LX-1 lung carcinoma on a daily 5 day schedule, caused a similar degree and similar kinetics of inhibition of RNR in LX-1 lung carcinoma at least for 12 h after administration. On the other hand, DMDC as well as 1-β-D-arabinofuranosyl-cytosine (ara-C), which is a well-known deoxycytidine analog and inhibits DNA polymerase α, did not inhibit RNR in LX-1 lung carcinoma at doses demonstrating antitumor activity. These results indicate that MDL 101,731 exhibited antitumor activity through inhibition of RNR activity in tumor cells *in vivo* and the mechanism of antitumor action of MDL 101,731 might be different from those of DMDC and ara-C, at least in part. [© 1998 Lippincott Williams & Wilkins.]

Key words: Antitumor effect, deoxycytidine analog, lung carcinoma, mechanism of action, MDL 101,731, ribonucleotide reductase.

Introduction

Ribonucleotide reductase (RNR) is one of the important enzymes involved in *de novo* deoxyribonucleotide synthesis and the level of RNR is elevated in tumor cells.[1,2] Therefore RNR may be a logical target for cancer chemotherapy. Currently, hydroxyurea and gemcitabine are clinically useful drugs which inhibit RNR.[3,4] Hydroxyurea has been used for treatment of chronic myelogenous leukemia and the myeloproliferative syndromes,[5] and has also been shown to be effective as a radiation sensitizer.[6] Gemcitabine, which also inhibited DNA polymerase α,[7] was effective against human solid tumors in nude mice models and in the clinic.[8–12] It was suggested that RNR inhibitory activity of gemcitabine contributed to its antitumor activity, both directly and indirectly.[4,13] The inhibition of RNR reduced deoxyribonucleoside triphosphate (dNTP) pools, leading to inhibition of DNA synthesis.[4] The reduction of dNTP pools may also augment inhibition of DNA polymerase α by gemcitabine, a mechanism which is based on the reduction of deoxycytidine triphosphate (dCTP), a natural substrate competing with gemcitabine for DNA polymerase α inhibition.[13]

(*E*)-2′-Deoxy-2′-(fluoromethylene)cytidine (MDL 101, 731) and 2′-deoxy-2′-methylidenecytidine (DMDC) are new deoxycytidine analogs which show potent antitumor activity against several human tumor models.[14–18] We previously showed that MDL 101,731 irreversibly inhibited human RNR in HeLa S3 human cervical carcinoma cells.[19] Baker *et al.*[20] also showed that DMDC irreversibly inhibits RNR from *Escherichia coli*. Recently, however it has been reported that DMDC does not inhibit RNR from intact L1210 murine leukemia cells at a concentration showing growth inhibition.[21] Thus, although MDL 101,731 was confirmed to inhibit human RNR in a cell-based system,[19] it was not clear whether MDL 101,731 inhibited RNR of tumor cells at doses demonstrating antitumor activity *in vivo*.

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In this study, we examined the relationship between the antitumor activity and RNR inhibitory activity of MDL 101,731 to elucidate the contribution of RNR inhibitory activity of MDL 101,731 to its antitumor efficacy *in vivo*.

Materials and methods

Reagents

MDL 101,731 and DMDC were provided by Hoechst Marion Roussel (Cincinnati, OH). 1-β-D-arabinofuranosylcytosine (ara-C) was purchased from Nippon Shinyaku Pharmaceutical (Kyoto, Japan). [^{3}H]Cytidine 5'-diphosphate (CDP, sp. act. 17.7 Ci/mmol) was purchased from Amersham (Little Chalfont, UK). All other reagents were obtained from Sigma (St Louis, MO).

Animals and tumors

LX-1 human lung carcinoma was maintained by s.c. implantation in adult male BALB/c *nu/nu* mice. BALB/c *nu/nu* mice were purchased from Clea Japan (Tokyo, Japan). All animals were kept pathogen free and were provided with food and water *ad libitum*, and used at 6-12 weeks old. LX-1 human lung carcinoma was supplied by Central Institute for Experimental Animals (Kawasaki, Japan).

Evaluation of antitumor activity

LX-1 lung carcinoma (8 mm^3 fragment) was implanted in BALB/c *nu/nu* mice s.c. When the tumor volume reached 50-300 mm^3 (day 0), MDL 101,731 was administered daily for 5 days by the i.v. or p.o. route. Tumor volume was calculated as: tumor volume (mm^3) = $L \times W^2/2$, where L and W are the length and width of the tumor. The drug efficacy against LX-1 lung carcinoma was expressed as the mean V/V_0 value against that of the control group, where V is the tumor volume at the day of evaluation and V_0 is the tumor volume at the day of initial drug treatment. The criteria of 'effective' was a T/C value of 0.5 and less, where T is the mean V/V_0 value of the drug-treated group and C is the mean V/V_0 value of the control group, and the statistical significance determined by the Mann-Whitney U-test ($p < 0.01$, one-side).[22] Experiments were conducted with five mice in a group.

RNR assay of tumor xenograft (*ex vivo*)

Fragments (8 mm^3) of LX-1 human lung carcinoma were implanted s.c. into BALB/c *nu/nu* mice and when the mean tumor volume reached 500 mm^3, MDL 101,731 was administered only once by the i.v. or p.o. route. After the administration, mice were sacrificed at the indicated time points and the tumors were collected. The crude enzyme containing RNR was extracted from the tumors with 20 mM hydroxyethylpiperidine ethanesulfonate (HEPES) buffer, pH 7.4, containing 2 mM dithiothreitol (DTT), 2 mM magnesium chloride and 1 mM phenylmethylsulfonyl fluoride. The crude enzyme was incubated with 0.65% streptomycin sulfate and after centrifugation brought to 50% saturation by the addition of $(NH_4)_2SO_4$. After centrifugation, the precipitate was dissolved in 50 mM HEPES buffer, pH 7.4, containing 2 mM DTT and used as the enzyme extract. RNR activity was measured according to the method of Cohen *et al.*[23] with slight modifications which we previously reported.[19]

Results

Antitumor activity of MDL 101,731

Table 1 shows the antitumor activity of MDL 101,731 and other deoxycytidine analogs at the daily 5 day administration schedule. MDL 101,731 exhibited potent antitumor activity against LX-1 human lung carcinoma at the dose of 5-15 mg/kg/day by i.v. administration. A similar degree of antitumor activity was obtained by p.o. administration of MDL 101,731 at doses of 10-30 mg/kg/day. In nude mice, the maximum-tolerated dose (MTD) of MDL 101,731 at the daily 5 day administration schedule was 10-15 mg/kg/day for the i.v. route and 20-30 mg/kg/day for the p.o. route. Therefore, equi-potent antitumor activity was observed at the equi-toxic dose of the i.v. and p.o. routes, respectively. DMDC also showed antitumor activity against LX-1 lung carcinoma at the MTD. In the case of ara-C, antitumor effect was obtained at the MTD, but the activity was somewhat low compared to MDL 101,731 and DMDC.

Inhibition of RNR activity by MDL 101,731 in ex vivo

MDL 101,731 inhibited RNR activity in LX-1 lung carcinoma by both i.v. and p.o. administration (Figure 1). Both 15 mg/kg of i.v. and 30 mg/kg of p.o.

Table 1. Antitumor activity of MDL 101,731 and other deoxycytidine analogs against LX-1 human lung carcinoma

Drug	Dose (mg/kg/day)	Route	T/C minimum (on day)	Maximum body weight change (%)[a]	Mortality
MDL 101,731	5	i.v.	0.15 (11)[b]	−5.8	0/5
	10	i.v.	0.04 (14)[b]	−3.3	0/5
	15	i.v.	0.04 (14)[b]	−3.2	0/5
	10	p.o.	0.08 (14)[b]	−3.6	0/5
	20	p.o.	0.04 (11)[b]	−6.8	0/5
	30	p.o.	0.04 (11)[b]	−7.1	1/5
DMDC	250	i.v.	0.13 (15)[b]	−19.2	0/5
Ara-C	150	i.v.	0.44 (15)[b]	−17.5	0/5

BALB/c nu/nu mice were implanted s.c. with an 8 mm^3 fragment of LX-1 lung carcinoma. When tumor volume reached 50–300 mm^3 (day 0), MDL 101,731 and other deoxycytidine analogs were administered daily for 5 days by the i.v. or p.o. route.
[a]Body weight change from initial values.
[b]Significantly different from the non-treated control group by Mann–Whitney U-test ($p < 0.01$).

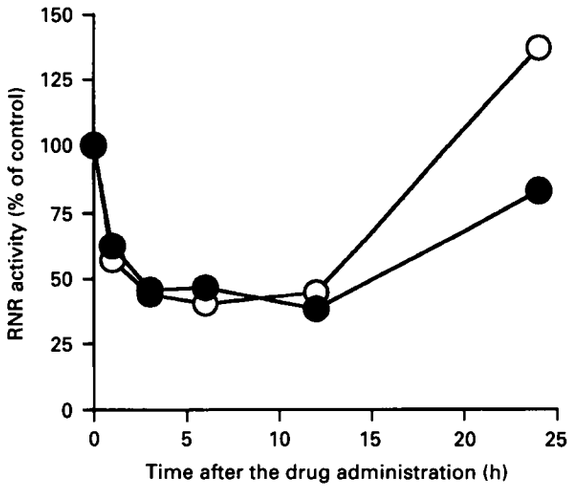

Figure 1. Time course of RNR activity in LX-1 human lung carcinoma treated with MDL 101,731. Fragments (8 mm^3) of LX-1 lung carcinoma were implanted s.c. into BALB/c nu/nu mice and when the mean tumor volume reached 500 mm^3, MDL 101,731 was administered once by the i.v. (●; 15 mg/kg) or p.o. (○; 30 mg/kg) route. Thereafter the tumor tissues were collected at the indicated time points and the RNR activity was measured as described in Materials and methods.

administration of MDL 101,731, which showed the same antitumor activity against LX-1 lung carcinoma, caused almost a similar degree of inhibition of RNR activity in LX-1 lung carcinoma at least for 12 h, though a rebound in RNR activity was observed by the p.o. route at 24 h after administration. On the other hand, DMDC and ara-C did not show any inhibition of RNR activity in LX-1 human lung carcinoma at the MTDs demonstrating antitumor activity (Table 2). At

Table 2. RNR inhibition in LX-1 human lung carcinoma by MDL 101,731 and other deoxycytidine analogs

Drug	Dose (mg/kg)	Route	RNR activity[a] (nmol/h mg protein)	Percent of control
MDL 101,731	5	i.v.	0.45	53.6
	15	i.v.	0.32	38.1
DMDC	250	i.v.	1.13	134.5
Ara-C	150	i.v.	0.90	107.1

Fragments (8 mm^3) of LX-1 lung carcinoma were implanted s.c. into BALB/c nu/nu mice and when the mean tumor volume reached 500 mm^3, MDL 101,731, DMDC or ara-C was administered once by the i.v. route. The tumors were collected at 6 h after administration and the RNR activity was measured as described in Materials and methods.
[a]RNR activity of non-treated LX-1 lung carcinoma (control) was 0.84 nmol/h mg protein.

the lowest dose of 5 mg/kg (i.v.) MDL 101,731 showed a similar degree of antitumor activity to that of DMDC (Table 1) and a clear inhibition of RNR activity was observed in the tumors in MDL 101,731-treated mice (Table 2).

Discussion

MDL 101,731 is a new deoxycytidine analog designed as a mechanism-based inhibitor of RNR,[24] and has recently been reported to show antitumor activity against human tumors such as breast, prostate and colon carcinoma xenografted in nude mice.[14–17]

Although MDL 101,731 was designed as an inhibitor of RNR, it inhibited DNA polymerase α as well as RNR.[25] In addition, it remains unknown whether inhibition of RNR activity by MDL 101,731 contributed to its antitumor effect. It was reported that

DMDC, one of the deoxycytidine analogs which inhibited RNR from *E. coli*,[20] did not inhibit RNR activity in L1210 murine leukemia cells at a concentration demonstrating tumor growth inhibition.[21] Therefore, we tried to examine the relationship between antitumor activity and RNR inhibitory activity of MDL 101,731 using LX-1 human lung carcinoma which was highly sensitive to this drug. MDL 101,731 inhibited RNR activity in LX-1 lung carcinoma at a dose demonstrating antitumor activity (Tables 1 and 2). In addition, i.v. and p.o. administration of MDL 101,731 clearly inhibited RNR activity to a similar degree at doses demonstrating almost the same antitumor activity, and the RNR inhibition by MDL 101,731 was maintained for at least 12 h after administration (Table 1 and Figure 1). These results indicate that the antitumor activity of MDL 101,731 can be correlated with its RNR inhibitory activity. On the other hand, DMDC as well as ara-C, which is also a deoxycytidine analog and is known to inhibit DNA polymerase α,[26] did not inhibit RNR in LX-1 lung carcinoma at the doses showing antitumor activity (Tables 1 and 2). Although DMDC was reported to have a RNR inhibitory activity,[20] the RNR inhibitory activity of DMDC might not contribute to its antitumor activity *in vivo*. When MDL 101,731 was administered on a daily schedule, the RNR activity of tumor cells seemed to be suppressed continuously, which in turn would lead to strong suppression of tumor growth. On the other hand, this marked inhibitory activity of RNR by the drug might possibly cause side effects on some rapidly growing normal tissues such as bone marrow cells and gastrointestinal mucous cells. Therefore it is important to determine the optimal administration schedule(s) that shows good antitumor effect but lesser side effect by considering the RNR inhibition pattern of MDL 101,731 in tumor cells and normal cells, because different sensitivities and different recovery profiles may be expected between normal and tumor cells.

DNA polymerase α is another action point of MDL 101,731, like gemcitabine and DMDC.[7,25,27] The inhibition pattern of DNA polymerase α by MDL 101,731 was similar to those of gemcitabine and DMDC which inhibit DNA polymerase α by their triphosphated form. In addition, RNR inhibition by MDL 101,731 also plays an important role in DNA polymerase α inhibition. The inhibition of RNR by MDL 101,731 might augment DNA polymerase α inhibition in tumor cells by reducing dCTP, a natural substrate competing with the triphosphate form of MDL 101,731 for DNA polymerase α inhibition. Thus, the strong inhibition of RNR by MDL 101,731 might contribute to its antitumor activity directly and indirectly.

Although we need some additional studies to elucidate a possible contribution of DNA polymerase α inhibitory activity of MDL 101,731 to the exact antitumor mechanism of this drug, our findings in this study show that the RNR inhibitory activity of MDL 101,731 surely contributes to its potent antitumor activity *in vivo*.

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