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# Metabolism and ribonucleotide reductase inhibition of (*E*)-2′-deoxy-2′-(fluoromethylene)cytidine, MDL 101,731, in human cervical carcinoma HeLa $S_3$ cells

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**Abstract** (*E*)-2′-Deoxy-2′-(fluoromethylene)cytidine, MDL 101,731, has shown potent antitumor activity against various human xenograft models. *Purpose*: The purpose of this study was to elucidate the mechanism of the antitumor activity of MDL 101,731 against human carcinoma cells through investigating metabolism and the target enzyme of MDL 101,731. *Methods*: In respect of the intracellular metabolism of MDL 101,731, the effect on enzymes in the pyrimidine salvage pathway and the intracellular metabolites of MDL 101,731 were investigated. In respect of the target enzyme, the effect on intracellular deoxyribonucleoside triphosphate (dNTP) pools and the inhibition of the enzyme activity were investigated. *Results*: MDL 101,731 which shows antiproliferative activity against human cervical carcinoma HeLa $S_3$ cells at nanomolar concentrations ($IC_{50}$, 30–50 n*M*), was hardly metabolized to (E)-2′-deoxy-2′-(fluoromethylene)uridine (FMdU) which had no antiproliferative activity below 100 μ*M* because of resistance to human cytidine deaminase. MDL 101,731 showed low activity against murine lymphocytic leukemia $P388^R$ cells (Ara-C-resistant cells) which contained lower deoxycytidine kinase activity than parental P388 cells. In addition, the antiproliferative activity of MDL 101,731 against HeLa $S_3$ cells was reversed by deoxycytidine. Studies of the intracellular metabolism of $^3$H-MDL 101,731 demonstrated that it was rapidly metabolized to the diphosphate and the triphosphate forms without the other metabolites in HeLa $S_3$ cells. A 3-h treatment with 0.1–10 μ*M* MDL 101,731 decreased intracellular dNTP pools. The recovery of dNTP pools decreased by treatment with 2 μ*M* MDL 101,731 was much slower than the recovery following treatment with 10 m*M* hydroxyurea, a reversible ribonucleotide reductase inhibitor. At a dose of 250 mg/kg, MDL 101,731 continuously inhibited ribonucleotide reductase activity up to 72 h in a HeLa $S_3$ xenograft model. *Conclusions*: This study suggests that the prolonged ribonucleotide reductase inhibition by rapidly activated metabolites of MDL 101,731 in part contributes to the potent antitumor activity of this drug against various xenografts.

**Key words** (*E*)-2′-Deoxy-2′-(fluoromethylene)cytidine Metabolism · Ribonucleotide reductase · Hydroxyurea · Cervical carcinoma

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## Introduction

Ribonucleotide reductase (RNR) is a critical enzyme responsible for the conversion of ribonucleoside diphosphates to the corresponding deoxyribonucleotides [15]. RNR inhibitors are known to inhibit effectively DNA synthesis and repair in mammalian cells [14, 30], presumably through inhibiting de novo synthesis of deoxyribonucleoside triphosphate (dNTP) pools participating in these processes. Hydroxyurea, a reversible RNR inhibitor, has been used for the treatment of chronic myelogenous leukemia and myeloproliferative syndromes [11], and also has been shown to be effective as a radiation sensitizer [4, 16]. Hydroxyurea exhibits limited activity against solid tumors [11]. Therefore, other RNR inhibitors have been investigated, but none has been proven to be superior to hydroxyurea [8, 10, 31] until the development of gemcitabine which is effective against solid tumors and leukemias [17, 19–22, 27].

(*E*)-2′-Deoxy-2′-(fluoromethylene)cytidine, MDL 101,731, was synthesized as a mechanism-based

inhibitor of RNR [24, 25]. MDL 101,731 has shown antiproliferative activity against a number of human tumor cell lines with 50% growth-inhibitory concentrations ($IC_{50}$s) of 5–100 n*M* and potent antitumor activity against a variety of murine tumor models as well as human tumors xenografted into nude mice [5, 6, 26]. MDL 101,731 is known to inhibit RNR from both *E. coli* and mice irreversibly [12, 25]. However, no information exists about human RNR inhibition by MDL 101,731 or the intracellular metabolism of the drug.

We focused on the intracellular metabolism, the effect on intracellular dNTP pools, and RNR inhibition by MDL 101,731 in human cervical carcinoma HeLa $S_3$ cells, aiming at elucidation of the mode of action of the potent antitumor activity of MDL 101,731 against human carcinoma cells.

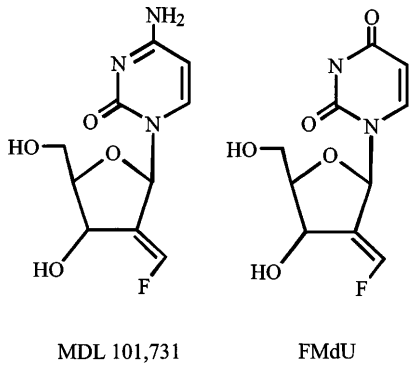
## Materials and methods

### Reagents

MDL 101,731 (Fig. 1), the monophosphate, the diphosphate and the triphosphate form of MDL 101,731, and (E)-2′-deoxy-2′-(fluoromethylene)uridine (FMdU) (Fig. 1) were provided by Hoechst Marion Roussel (Cincinnati, Ohio). $^3$H-MDL 101,731 (specific activity 23.0 Ci/mmol) was prepared by Tokai Research Laboratories (Ibaraki, Japan). 1–β–D-Arabinofuranosyl-cytosine (Ara-C), 1–β–D-arabinofuranosyl-uracil (Ara-U) and hydroxyurea were purchased from Sigma Chemical Co. (St. Louis, Mo.). $^3$H-Cytidine 5′-diphosphate (CDP, specific activity 17.7 Ci/mmol) was purchased from Amersham (Little Chalfont, UK.). All other reagents containing dNTP were purchased from Sigma Chemical Co.

### Cell lines and culture conditions

Human cervical carcinoma HeLa $S_3$ cells were maintained in minimum essential medium supplemented with 2 m*M* glutamine and 10% fetal bovine serum (Filtron, Brooklin, Australia) at 37 °C in an atmosphere containing 5% $CO_2$. The average volume of HeLa $S_3$ cells was 1.557 pl/cell. Murine lymphocytic leukemia P388 cells and $P388^R$ cells which showed about a 500-fold higher resistance to Ara-C than the parental P388 cells (these $P388^R$ cells were established by continuous intravenous Ara-C administration to mice bearing P388 cells intraperitoneally) were maintained in RPMI-1640 medium supplemented with 100 units penicillin, 100 μg/ml streptomycin, 20 μ*M* 2-mercaptoethanol and 10% fetal bovine serum. The HeLa $S_3$ xenografts were established by inoculating a suspension of at least $1–2 \times 10^6$ cells subcutaneously along the flank of male adult BALB/cAJcl-nu miced (Clea Japan, Tokyo, Japan), and they were then maintained in vivo.




MDL 101,731 FMdU

**Fig. 1** Structure of MDL 101,731 and FMdU

### In vitro antiproliferative activity

Cells were exposed to drugs for 72 h. Cell growth was determined using a microculture tetrazolium (MTT, Sigma Chemical Co.) assay described previously [1, 2]. The antiproliferative activity of the drugs is shown in terms of $IC_{50}$ values.

### Deaminase assay

Crude enzyme containing cytidine deaminase was extracted from HeLa $S_3$ cells with 10 m*M* phosphate buffer, pH 7.4 containing 1 m*M* dithiothreitol (DTT). The crude extract was brought to 75% saturation by the addition of $(NH_4)_2SO_4$ and then dissolved in phosphate-buffered saline containing 1 m*M* DTT after centrifugation. A 50 μl (4 mg/ml) aliquot of the enzyme extract was added to 200 μl 3 m*M* MDL 101,731 or 3 m*M* Ara-C with or without 0.25 m*M* tetrahydrouridine (THU), a cytidine deaminase inhibitor. This mixture was incubated for up to 8 h at 37 °C and then terminated by heating. After centrifugation, the supernatant was separated and quantitated by reversed-phase high-performance liquid chromatography (HPLC) using a Develosil ODS-HG-5 column (250 × 4.6 mm i.d.; Nomura Chemical, Aichi, Japan) with 10 m*M* phosphate buffer, pH 7.4, containing 5% methanol. Peaks were detected by absorption at 268 nm. THU completely inhibited deamination of both drugs.

### Determination of intracellular metabolites

HeLa $S_3$ cells were exposed to 10 μ*M* $^3$H-MDL 101,731 for up to 3 h and then the metabolites were extracted from the cells with 0.6 *N* trichloroacetic acid (TCA). The TCA-soluble extract was neutralized, separated and quantitated by ion-pair reversed-phase HPLC. The extract was loaded onto a Develosil ODS-HG-5 column and eluted with 200 m*M* ammonium phosphate buffer, pH 5.3, containing 5 m*M* tetrabutylammonium and 7% methanol. Peaks were detected by absorption at 270 nm and by measurement of radioactivity.

### Determination of dNTP pools

HeLa $S_3$ cells were exposed to 0.1–10 μ*M* MDL 101,731 for 3 h and then nucleoside triphosphates were extracted from the cells with 0.6 *N* TCA. The TCA-soluble cell extracts were neutralized and then the ribonucleoside triphosphates were degraded by periodate oxidation [18]. The extract was loaded onto a Partisil-10 SAX anion exchange column (250 × 4.6 mm i.d.; Whatman, Clifton, N.J.), separated and quantitated by HPLC analysis. Isocratic elution with 75% buffer A (0.005 *M* $NH_4H_2PO_4$, pH 2.9) and 25% buffer B (0.75 *M* $NH_4H_2PO_4$, pH 4.0) was maintained for 20 min at a flow rate of 2 ml/min. A linear gradient led to 31% buffer A and 69% buffer B over 23 min which was then maintained for 17 min. Peaks were detected by absorption at 270 nm.

### RNR activity

Crude enzyme containing RNR was extracted from HeLa $S_3$ xenografts with 20 m*M* hydroxyethylpiperidine ethanesulfonate (HEPES) buffer, pH 7.4, containing 2 m*M* DTT, 2 m*M* magnesium chloride and 1 m*M* 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 m*M* HEPES buffer, pH 7.4, containing 2 m*M* DTT and used as the enzyme extract.

RNR activity was determined according to the method of Cohen et al. [9] with slight modification. Briefly, 75 µl of the reaction mixture was composed of 50 m*M* HEPES buffer, pH 7.4, containing 5 m*M* ATP, 5 m*M* DTT, 5 m*M* $MgCl_2$, 10 m*M* sodium fluoride, 50 µ*M* CDP, 1.25 µ*Ci* $^3$ H-CDP and 500 µg of the enzyme extract. The mixture was incubated for 45 min at 37 °C and then terminated by heating. After treatment with 0.5 mg of *Crotalus adamenteus* venom (Sigma Chemical Co.) for 2 h, the cytidine and deoxycytidine formed were determined by thin-layer chromatography on a polyethyleneimine (PEI) cellulose plastic sheet which had been converted to the borate form as described by Schrecker et al. [28] using a solution of 0.5 m*M* ammonium formate, pH 4.7, containing 50% ethanol. The radioactivity corresponding to cytidine and deoxycytidine in the sheet was analyzed using a BAS-2000 system (Fuji Film, Tokyo, Japan).

## Results

### Antiproliferative activity of FMdU

The antiproliferative activity of MDL 101,731 and FMdU, which was obtained from MDL 101,731 by deamination, was determined by an MTT assay after a 72-h treatment (Fig. 2). MDL 101,731 showed a concentration-dependent growth inhibition against human cervical carcinoma HeLa $S_3$ cells giving an $IC_{50}$ value of about 30 n*M*. FMdU showed no growth inhibition up to 100 µ*M*.

### Resistance to cytidine deaminase

The principal catabolic form of MDL 101,731 can be assumed to be FMdU which is produced by cytidine deaminase. To investigate whether MDL 101,731 was deaminated by cytidine deaminase and converted to FMdU or not, MDL 101,731 was incubated with the extract from HeLa $S_3$ cells which had high cytidine deaminase activity for up to 8 h, and then the FMdU produced was measured by reversed-phase HPLC

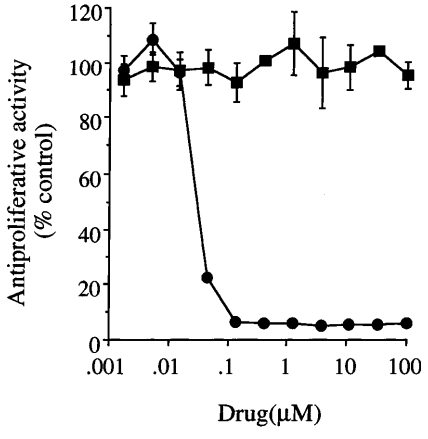



**Fig. 2** Antiproliferative activity of MDL 101,731 and FMdU against HeLa $S_3$ cells. Cells were exposed to 0.002–100 µ*M* MDL 101,731 (●) or FMdU (■) for 72 h. Cell growth was determined by the MTT assay. Values are the means ± SD of three separate experiments

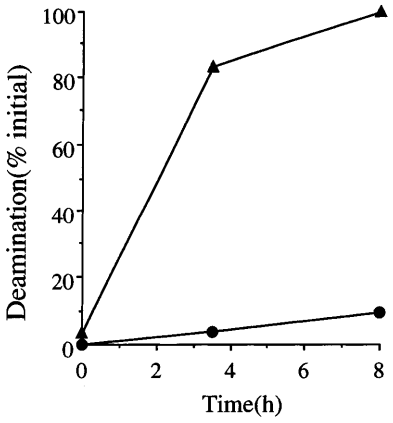



**Fig. 3** Deamination of MDL 101,731 and Ara-C by human cytidine deaminase. Extract from HeLa $S_3$ cells was inculated with 3 m*M* MDL 101,731 (●) or 3 m*M* Ara-C (▲) for up to 8 h. The FMdU or Ara-U produced was then separated and quantitated by reversed-phase HPLC. Values are the means of two separate experiments

(Fig. 3). Only 5% of the MDL 101,731 was converted to FMdU after a 3.5-h incubation, whereas at least 80% of Ara-C was converted to Ara-U through deamination. In addition, the conversion from MDL 101,731 to FMdU after an 8-h incubation was also at most 10%. This result demonstrated that MDL 101,731 was relatively resistant to inactivation by cytidine deaminase.

### Activation by deoxycytidine kinase

To investigate the anabolism of MDL 101,731 by deoxycytidine kinase, murine lymphocytic leukemia $P388^R$ cells which showed about a 500-fold higher resistance than the parental P388 cells to Ara-C were used. These cells showed about a threefold decrease in deoxycytidine kinase activity compared with the parental cells (P388 32.8 nmol/h per mg, $P388^R$ 12.1 nmol/h per mg). The antiproliferative activity of MDL 101,731 against P388 and $P388^R$ cells was determined by an MTT assay after a 72-h treatment. The $IC_{50}$ for P388 cells was 20 n*M*, whereas that of $P388^R$ cells was 2.7 µ*M*. The antiproliferative activity of MDL 101,731 against $P388^R$ cells was decreased at least 100-fold compared with that of MDL 101,731 against P388 cells.

We also investigated the ability of pyrimidine and purine nucleosides to rescue the antiproliferative activity of MDL 101,731 using an MTT assay after a 72-h treatment (Table 1). HeLa $S_3$ cells were incubated with MDL 101,731 alone or MDL 101,731 plus deoxyribonucleosides (deoxycytidine, deoxyuridine, deoxyadenosine, deoxyguanosine) or ribonucleoside (cytidine) each at 100 µ*M*. The $IC_{50}$ of MDL 101,731 alone was 30–40 n*M*. The addition of deoxycytidine resulted in a 24-fold decrease in the antiproliferative activity of MDL 101,731, but the other deoxyribonucleosides and ribonucleoside did not affect the activity of the drug.

**Table 1** Effects of pyrimidine and purine nucleosides on the antiproliferative activity of MDL 101,731. HeLa $S_3$ cells were exposed to MDL 101,731 alone or MDL 101,731 plus 100 μ*M* of each of the deoxyribonucleosides and ribonucleoside for 72 h. Cell growth was determined by the MTT assay. Values are the means of three separate experiments

| Nucleoside | Antiproliferative activity | |
|---|---|---|
| | $IC_{50}$ n*M* | Ratio |
| None | 28 | 1.0 |
| Deoxycytidine | 674 | 24.1 |
| Deoxyuridine | 43 | 1.5 |
| Deoxyadenosine | 62 | 2.2 |
| Deoxyguanosine | 49 | 1.8 |
| None | 43 | 1.0 |
| Cytidine | 57 | 1.4 |

These results suggest that MDL 101,731 is activated by deoxycytidine kinase.

## Intracellular metabolites

No information exists about the intracellular metabolism of MDL 101,731. HeLa $S_3$ cells were treated with 10 μ*M* $^3$H-MDL 101,731 for up to 3 h to investigate the intracellular metabolites (Fig. 4A,B). The determination of the metabolites was performed by ion-pair reversed-phase HPLC equipped with a radioactive flow detector. The presumed metabolites of MDL 101,731 are the monophosphate, the diphosphate and the triphosphate forms, and their standard samples eluted at 4.9 min, 7.4 min and 12.1 min, respectively. After a 15-min treatment with 10 μ*M* $^3$H-MDL 101,731, the $^3$H-diphosphate and the $^3$H-triphosphate forms were detected, but not $^3$H-MDL 101,731, the $^3$H-monophosphate form or the other $^3$H-metabolites containing FMdU. They were not detected even after 3 h of treatment. This result is consistent with resistance to cytidine deaminase of MDL 101,731 and activation by deoxycytidine kinase. The intracellular concentration of the $^3$H-diphosphate form increased linearly up to 3 h reaching 2 μ*M*, 4 μ*M* and 10 μ*M* after 15 min, 1 h and 3 h, respectively. The concentration of the triphosphate form also increased linearly up to 3 h reaching 10 μ*M*, 45 μ*M* and 145 μ*M* after 15 min, 1 h and 3 h, respectively.

## Effects on dNTP pools

The effect of MDL 101,731 on the intracellular dNTP pools was investigated in HeLa $S_3$ cells exposed to MDL 101,731 in the range 0.1–10 μ*M* for 3 h (Table 2). In untreated HeLa $S_3$ cells, the dCTP pool (30.7 ± 1.9 μ*M*) was the smallest compared with the TTP pool (185.9 ± 2.6 μ*M*), the dATP pool (114.9 ± 2.7 μ*M*), and the dGTP pool (47.6 ± 7.8 μ*M*). A 3-h treatment with MDL 101,731 induced a decrease in three

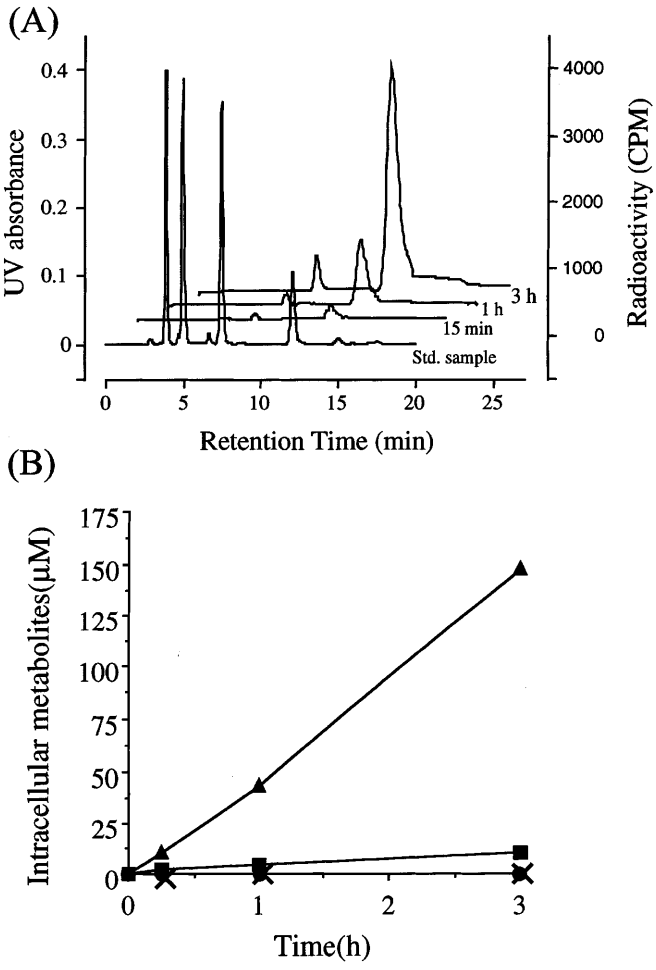



**Fig. 4A,B** Intracellular metabolites of $^3$H-MDL 101,731 in HeLa $S_3$ cells. Cells were exposed to 10 μ*M* $^3$H-MDL 101,731 for up to 3 h. The determination of the metabolites was performed by ion-pair reversed-phase HPLC equipped with a radioactive flow detector. **A** This is a representative chromatogram of three separate experiments. MDL 101,731, the monophosphate, the diphosphate and the triphosphate forms in the standard samples were eluted at 3.8 min, 4.9 min, 7.4 min, and 12.1 min, respectively. **B** The concentrations of intracellular metabolites of $^3$H-MDL 101,731 produced in HeLa $S_3$ cells was determined in terms of the radioactivity ( × MDL 101,731, ● monophosphate, ■ diphosphate, ▲ triphosphate). The average volume of HeLa $S_3$ cells was 1.557 pl/cell. Values are the means of two separate experiments

**Table 2** Effect of MDL 101,731 on dNTP pools in HeLa $S_3$ cells. Cells were exposed to 0.1–10 μ*M* MDL 101,731 for 3 h and then dNTP pools were extracted from cells, separated, and quantitated by HPLC analysis using an anion exchange column. Values are the means of three separate experiments. dNTP pools in untreated HeLa $S_3$ cells: dCTP pool (30.7 ± 1.9 μ*M*), TTP pool (185.9 ± 2.6 μ*M*), dATP pool (114.9 ± 2.7 μ*M*), dGTP pool (47.6 ± 7.8 μ*M*)

| MDL 101,731 (μ*M*) | % control | | | |
|---|---|---|---|---|
| | dCTP | TTP | dATP | dGTP |
| 0.1 | 46.6 | 89.0 | 48.1 | 54.8 |
| 1.0 | <42.3 | 136.8 | 10.6 | 27.1 |
| 10 | <42.3 | 162.8 | <10.4 | 25.4 |

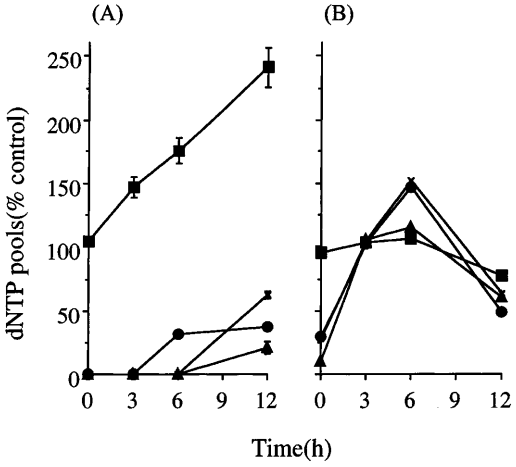



**Fig. 5A,B** Recovery of dNTP pools after treatment with MDL 101,731 and hydroxyurea in HeLa $S_3$ cells. Cells were exposed to 2 μ*M* MDL 101,731 **A** or 10 m*M* hydroxyurea **B** for 3 h, washed and placed in fresh medium (time 0 h). dNTP pools (● dCTP, ■ TTP, ▲ dATP, × dGTP) were extracted from cells at the indicated times and separated and quantitated by HPLC analysis using an anion exchange column. **A** Values are the means ± SD of three separate experiments. **B** Values are the means of two separate experiments

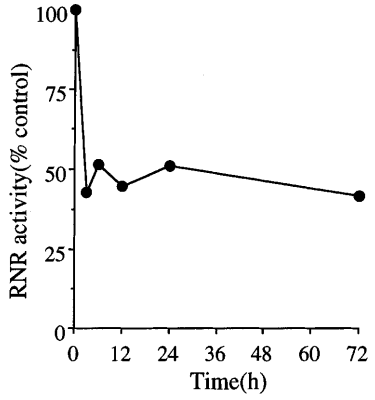



**Fig. 6** Prolonged inhibition of RNR activity in HeLa $S_3$ xenograft. MDL 101,731 was administered intravenously at 250 mg/kg into HeLa $S_3$ cells-bearing mice. After taking the tumor from the mice at 3 h, 6 h, 12 h, 24 h, and 72 h, RNR activity in the tumor was determined ex vivo using $^3$H-CDP as the RNR substrate. Values are from a single experiment

dNTP pools (dCTP, dATP and dGTP) but not the TTP pool. This effect was apparent at 0.1 μ*M* MDL 101,731 which decreased the level to about 50% of control levels. Further decreases in the three dNTP pools were observed with 1 μ*M* and 10 μ*M* MDL 101,731, whereas the TTP pool was increased to 135% and 160% of control levels, respectively, with these concentrations.

We compared the recovery of the dNTP pools decreased by MDL 101,731 and the RNR inhibitor hydroxyurea in HeLa $S_3$ cells. The effect of 1–10 μ*M* MDL 101,731 treatment for 3 h on dNTP pools was qualitatively and quantitatively similar to that of 10 m*M* hydroxyurea. HeLa $S_3$ cells were incubated with 2 μ*M* MDL 101,731 or 10 m*M* hydroxyurea for 3 h. They were then washed and placed in fresh drug-free medium (Fig. 5A,B). After a 3-h treatment, both drugs apparently induced a decrease in three dNTP pools but not the TTP pool. By 3-h after placing in the fresh drug-free medium, dNTP pools decreased by hydroxyurea had recovered to control levels, whereas the dNTP pools decreased by MDL 101,731 had not recovered after a 12-h treatment. The dCTP and dATP pools decreased by the drug did not recover to control levels even after 24-h of treatment (data not shown). This result suggests that MDL 101,731 inhibited RNR irreversibly whereas the effect of hydroxyurea was reversible.

### Prolonged inhibition of RNR activity in HeLa $S_3$ xenografts

To investigate whether MDL 101,731 would inhibit human RNR, a 250 mg/kg dose of MDL 101,731, the maximum tolerated dose in a twice weekly schedule (every 3 days × 4), was administered intravenously into the HeLa $S_3$ cell-bearing mice. The RNR activity in the HeLa $S_3$ xenografts was determined using an ex vivo assay using $^3$H-CDP as the substrate after removing the tumors from the mice at 3 h, 6 h, 12 h, 24 h and 72 h (Fig. 6). MDL 101,731 inhibited the RNR activity in the tumors by 60% within 3 h and the inhibition continued up to 72 h. This prolonged inhibition might in part have been due to irreversible inhibition of RNR.

## Discussion

One of the aims of this investigation was to elucidate the intracellular metabolism of MDL 101,731. We first investigated the inactivation of MDL 101,731 by cytidine deaminase. MDL 101,731 was hardly metabolized to FMdU which showed no antiproliferative activity because of resistance to human cytidine deaminase (Fig. 3). This was consistent with the result that FMdU was not observed in HeLa $S_3$ cells treated with $^3$H-MDL 101,731 (Fig. 4A,B) and that MDL 101,731 showed potent antiproliferative activity against HeLa $S_3$ cells which contain high cytidine deaminase activity (Fig. 2). This contrasts with the effects of the related compound gemcitabine, which is deaminated as efficiently as Ara-C [27]. Therefore, these results indicate that MDL 101,731 could not be inactivated in human liver, kidney or solid tumors which contain high cytidine deaminase activity [23]. Therefore MDL 101,731 may achieve and maintain effective concentrations in humans. We are also investigating the deamination of MDL 101,731 by deoxycytidine monophosphate deaminase, another enzyme that plays an important role in the catabolic pathway of deoxycytidine analogs such as gemcitabine [17].

We next investigated the possibility of activation of MDL 101,731 by deoxycytidine kinase, because MDL

101,731 is an analog of deoxycytidine (Fig. 1). The antiproliferative activity of MDL 101,731 against HeLa $S_3$ cells was reversed by deoxycytidine, but not deoxyuridine, deoxyadenosine, deoxyguanosine or cytidine (Table 1). In addition, the antiproliferative activity of MDL 101,731 against $P388^R$ cells which contain lower deoxycytidine kinase activity than the parental P388 cells was decreased 100-fold compared with the activity of MDL 101,731 against P388 cells. These results suggest that MDL 101,731 may be activated by deoxycytidine kinase, as well as Ara-C and gemcitabine [7, 13].

Preliminary evidence using the in vitro cell-free deoxycytidine kinase assay demonstrated that the substrate specificity (Vmax/Km) of MDL 101,731 for human deoxycytidine kinase was decreased tenfold compared with that of Ara-C and gemcitabine. However, the intracellular concentrations of MDL 101,731 metabolites were 16-fold higher than the extracellular concentrations after 3 h of treatment in vivo (Fig. 4B). This inconsistency between in vitro and in vivo activation might be explained by the decrease in the intracellular dCTP pool (Table 2), which is a negative regulator of deoxycytidine kinase [29]. It is reasonable to assume that the decrease in the dCTP pool led to the efficient accumulation of a large quantity of metabolites of MDL 101,731 in vivo (Fig. 4B). This is very similar to the activation of gemcitabine [18, 19]. The metabolites of Ara-C did not accumulate more than those of MDL 101,731 in HeLa $S_3$ cells partially because the dCTP pool did not decrease [17, 27]. These results indicate that, for the rapid accumulation of large quantities of MDL 101,731 metabolites, it would be important to decrease the intracellular dCTP pool.

The other aim of this investigation was to elucidate the effects on intracellular dNTP pools and RNR-inhibition of MDL 101,731 in human cells. MDL 101,731 induced decreases of the dATP and dGTP pools as well as of the dCTP pool (Table 2). This effect was particularly marked at 1 μ*M* and 10 μM MDL 101,731. Treatment with 1–10 μ*M* MDL 101,731 for 3 h resulted in the accumulation of 1 μmol or more of the diphosphate form of MDL 101,731. This result is consistent with the result that the diphosphate form of MDL 101,731, but not the triphosphate, can inhibit purified human RNR at sub-micromolar concentrations of Ki (manuscript in preparation). In addition, preliminary evidence indicated that the diphosphate of MDL 101,731 inhibited RNR more potently than the diphosphate of gemcitabine and 2′-deoxy-2′-methylenecytidine, which are potent mechanism-based inhibitors of RNR.

Recovery of dNTP pools (Fig. 5A, B) and inhibition of RNR activity in xenografts (Fig. 6) after MDL 101,731 treatment demonstrated that RNR-inhibition of MDL 101,731 was long lasting. This prolonged inhibition might presumably be related to the irreversibility of RNR inhibition, suggesting that RNR is inhibited by a mechanism-based action of MDL 101,731 as is the case for gemcitabine and 2′-deoxy-2′-methylenecytidine [3]. This study suggests that the prolonged inhibition of RNR by MDL 101,731 would, in part, contribute to the potent antitumor activity of the drug against xenografts [5, 6, 26].

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