

## Schedule-Dependent Drug Effects of Oral 5-Iodo-2-Pyrimidinone-2'-Deoxyribose as an *In vivo* Radiosensitizer in U251 Human Glioblastoma Xenografts

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**Abstract Purpose:** 5-Iodo-2-pyrimidinone-2'-deoxyribose (IPdR) is an oral prodrug of 5-iodo-2'-deoxyuridine (IUdR), an *in vitro/in vivo* radiosensitizer. IPdR can be rapidly converted to IUdR by a hepatic aldehyde oxidase. Previously, we found that the enzymatic conversion of IPdR to IUdR could be transiently reduced using a once daily (q.d.) treatment schedule and this may affect IPdR-mediated tumor radiosensitization. The purpose of this study is to measure the effect of different drug dosing schedules on tumor radiosensitization and therapeutic index in human glioblastoma xenografts.

**Experimental Design:** Three different IPdR treatment schedules (thrice a day, t.i.d.; every other day, q.o.d.; every 3rd day, q.3.d.), compared with a q.d. schedule, were analyzed using athymic nude mice with human glioblastoma (U251) s.c. xenografts. Plasma pharmacokinetics, IUdR-DNA incorporation in tumor and normal proliferating tissues, tumor growth delay following irradiation, and body weight loss were used as end points.

**Results:** The t.i.d. schedule with the same total daily doses as the q.d. schedule (250, 500, or 1,000 mg/kg/d) improved the efficiency of IPdR conversion to IUdR. As a result, the percentage of IUdR-DNA incorporation was higher using the t.i.d. schedule in the tumor xenografts as well as in normal small intestine and bone marrow. Using a fixed dose (500 mg/kg) per administration, the q.o.d. and q.3.d. schedules also showed greater IPdR conversion than the q.d. schedule, related to a greater recovery of hepatic aldehyde oxidase activity prior to the next drug dosing. In the tumor regrowth assay, all IPdR treatment schedules showed significant increases of regrowth delays compared with the control without IPdR (q.o.d., 29.4 days; q.d., 29.7 days; t.i.d., 34.7 days; radiotherapy alone, 15.7 days). The t.i.d. schedule also showed a significantly enhanced tumor growth delay compared with the q.d. schedule. Additionally, the q.o.d. schedule resulted in a significant reduction in systemic toxicity.

**Conclusions:** The t.i.d. and q.o.d. dosing schedules improved the efficiency of enzymatic activation of IPdR to IUdR during treatment and changed the extent of tumor radiosensitization and/or systemic toxicity compared with a q.d. dosing schedule. These dosing schedules will be considered for future clinical trials of IPdR-mediated human tumor radiosensitization.

5-Iodo-2-pyrimidinone-2'-deoxyribose (IPdR) is a thymidine analogue that was originally synthesized as an antiviral agent, based on the hypothesis that nucleosides without an amino- or keto-group at position 4 of the pyrimidine ring could be used as a substrate of viral thymidine kinase but not mammalian thymidine kinase (1, 2). However, the same investigators

subsequently found that IPdR could be converted to 5-iodo-2'-deoxyuridine (IUdR) by an aldehyde oxidase that was mainly localized to rodent liver (3). This reaction is shown in Fig. 1, and has been recently reviewed (4).

IUdR is also a halogenated thymidine analogue and has been recognized as an *in vitro/in vivo* radiosensitizer since the 1960s (5). The mechanism of radiosensitization is most likely related to the generation of highly reactive free radicals by ionizing radiation (IR) from IUdR incorporated into DNA, resulting in enhanced ionizing radiation-induced DNA strand breaks. The amount of thymidine replacement by IUdR in DNA is generally recognized to correlate directly with the extent of radiosensitization. A major drawback of IUdR as a clinical radiosensitizer is DNA incorporation in rapidly proliferating normal tissues, which results principally in myelosuppression and gastrointestinal toxicities. In preclinical animal studies, the percentage of IUdR-DNA incorporation of bone marrow and intestine were significantly (two to three times) higher than that of human tumor xenografts using continuous infusions of IUdR (6–8). For this reason, primary or metastatic tumors

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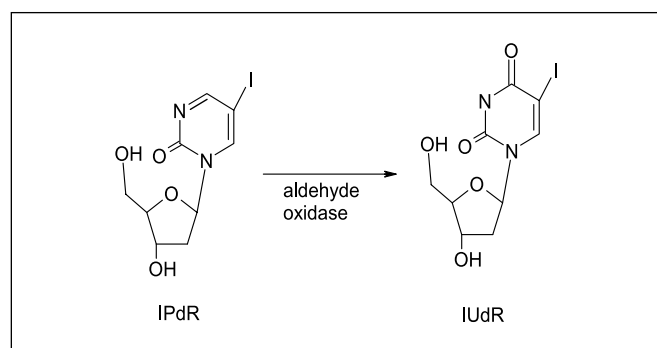


Fig. 1. Molecular structures of IPdR and IUdR.

of the brain or liver and high-grade sarcomas were selected as clinical targets for IUdR-mediated radiosensitization as these tumors are surrounded by nonproliferating normal tissues. However, acute toxicities to bone marrow and intestine still limited the duration and total dose of continuous infusions of IUdR, which consequently limited radiosensitization (9–15).

Because IPdR per se has shown much less systemic toxicity than IUdR and can be converted to IUdR *in vivo*, we have recently focused on IPdR as an oral prodrug for IUdR-mediated human tumor radiosensitization. In our recent publications, we have shown that normal liver tissue in rodents and non-rodent animals rapidly converts IPdR to IUdR *in vitro* and *in vivo*, as well as showing similar IPdR metabolism using extracts of normal human liver *in vitro* (8, 16). Oral administration of IPdR can significantly improve the therapeutic index, compared with continuous infusion IUdR using several different human tumor xenograft models (8, 17–19). The extent (%) of IUdR-DNA incorporation in the small intestine and bone marrow were remarkably reduced with a similar or superior percentage of IUdR-DNA incorporation in s.c. human tumor xenografts using oral IPdR given once daily (q.d.) compared with the maximum tolerated dose of IUdR as a continuous infusion (8, 17, 19). Athymic nude mice and rats can tolerate oral IPdR administration given q.d. up to 1,500 mg/kg/d for 14 to 28 days without marked systemic toxicity as measured by body weight change (18).<sup>3</sup> However, we also found that the rate-limiting enzyme of IPdR treatment, hepatic aldehyde oxidase, can be transiently saturated following high doses ( $\geq 1,000$  mg/kg) of IPdR administration (18).

We have already planned the initial phase I clinical trial using oral IPdR at a starting dose of 85 mg/m<sup>2</sup> q.d.  $\times$  28 days under a National Cancer Institute Rapid Access to Intervention Development grant #197 (T. Kinsella, PI). However, based on previous observations, the hepatic conversion of IPdR to IUdR may limit dose escalation of IPdR given once daily in spite of the observed relatively mild myelosuppression and gastrointestinal toxicity in rodents and ferrets (8, 16). The aim of this preclinical study is to seek alternative IPdR treatment schedules that lead to improved IPdR conversion and subsequent higher IUdR-DNA incorporation into tumor tissues compared with the q.d. administration schedule used in our prior experimental studies (8, 16–19). In this study, we test two alternative dosing schedules; one is a fixed daily dose but with more frequent administration, where IPdR is given thrice a day (t.i.d.). The

other method is to administer a fixed dose with longer intervals between doses (every other day, q.o.d. or every 3rd day, q.3.d.). We show that these alternative dosing schedules can improve the efficiency of IPdR conversion to IUdR, which subsequently affect the degree of tumor radiosensitization as well as systemic toxicity. The therapeutic index is determined by a comparison of the percentage of IUdR-DNA incorporation in the U251 human glioblastoma xenografts to two normal proliferating tissues (intestine and bone marrow) as well as systemic toxicity (body weight loss).

## Materials and Methods

**Drugs and chemicals.** IPdR was synthesized and obtained from SuperGen, Inc. (Pleasanton, CA). IPdR was suspended daily in 10% gum arabic in PBS (vehicle). Sodium acetate, potassium phosphate, acetonitrile, and methanol [high-performance liquid chromatography (HPLC) grade] were purchased from Fisher Scientific (Pittsburgh, PA). Other chemicals were purchased from Sigma Co. (St. Louis, MO) unless otherwise indicated.

**Cell culture and tumor xenograft.** The U251 human glioblastoma cell line was maintained in DMEM supplemented with 10% fetal bovine serum, L-glutamine, and penicillin/streptomycin at 37°C in a humidified 10% CO<sub>2</sub> atmosphere. Exponentially growing tumor cells were harvested from cell culture plates by trypsinization. Three  $\times 10^6$  cells in 50  $\mu$ L PBS were injected into the s.c. tissue of the dorsal flank of 5- to 7-week-old athymic nude mice. Each group consisted of equal numbers of male and female mice. The animals were housed in 12-hour lights on/off cycles under laminar flow ventilation with food and water provided ad libitum. Two dimensions of tumor xenografts were measured daily. Once the tumor dimensions reached 25 to 30 mm<sup>2</sup> (typically 7–10 days after tumor cell injection), drug treatments were begun.

**5-Iodo-2-pyrimidinone-2'-deoxyribose administration and blood/tissue sampling.** Groups of mice were given IPdR by a gastric gavage at the various dose schedules as indicated. For control mice, the same volume of vehicle was given once daily. Weights of the mice and the tumor dimensions were monitored daily to assess general health conditions. On the last day of the drug treatment,  $\sim 40$   $\mu$ L of blood was drawn and collected in Microvette tubes CB300 (Sarstedt, Germany) by a saphenous vein puncture at 0.25, 0.5, 1, 2, and 4 hours after the last dose of IPdR. Then, 8 hours after the last dose of IPdR, mice were anesthetized by an i.p. injection of ketamine and euthanized by exsanguination with a cardiac puncture. Plasma was separated from whole blood by centrifugation at 5,000  $\times g$  at 4°C for 10 minutes. Small intestine, femur, liver, and s.c. tumor xenograft were harvested immediately after exsanguination, and then frozen using dry ice. Plasma and tissue samples were stored at  $-80^\circ\text{C}$  until analysis.

**5-Iodo-2'-deoxyuridine-DNA incorporation assay in tumor and normal tissues.** The percentage of IUdR-DNA incorporation was determined according to the method of Belanger et al. (20), with minor modifications as previously described (17). At the time of analysis, the tissues were thawed and minced in PBS. The samples were homogenized using a sonic dismembrator followed by DNA isolation and enzymatic hydrolysis. A solution containing nucleosides and the analogue were analyzed by HPLC with a reversed-phase column. HPLC analysis was done using a Waters System controller 600E, Autosampler 717, Multiwavelength detector 400E, and a 3.9  $\times$  300 mm  $\mu$ Bondapak C<sub>18</sub> reversed-phase column (Waters Co., Milford, MA). The typical retention times of TdR and IUdR were 6 and 9 minutes, respectively. The percentage of IUdR-DNA incorporation was calculated by the formula:

$$\frac{\text{IUdR}}{\text{IUdR} + \text{TdR}} \times 100.$$

**Plasma drug concentration analysis.** Extraction of the nucleoside analogues was done as we published previously (17). Briefly, 20  $\mu$ L of

<sup>3</sup> Unpublished data.

100  $\mu\text{mol/L}$  5-bromodeoxyuridine was added to 20  $\mu\text{L}$  of plasma as an internal standard. Two volumes of ice-cold acetonitrile were then added and vortexed vigorously. The mixture was placed on ice for 30 minutes, and then centrifuged at  $1,000 \times g$  at  $4^\circ\text{C}$  for 5 minutes. The upper (acetonitrile) layer was recovered in a new tube, and dried under reduced pressure. The samples were stored at  $-80^\circ\text{C}$  until HPLC analysis. The samples were reconstituted in distilled water and injected into the reversed-phase HPLC system. Peaks were detected by UV absorption at 335 nm for IPdR and at 290 nm for bromodeoxyuridine and IUdR. Typical elution times of bromodeoxyuridine, IUdR, and IPdR were 17, 21, and 22 minutes, respectively. Plasma IPdR and IUdR areas under the concentration-time curve (AUC) were estimated by a linear trapezoidal method. When the concentration of a drug in plasma at 8 hours was not 0,  $\text{AUC}_{0-\infty}$  was estimated by combining  $\text{AUC}_{0-8}$  using the trapezoidal method and  $\text{AUC}_{8-\infty}$  estimated by the following equation (21):

$$\text{AUC}_{8-\infty} = \frac{C^*}{\lambda}$$

where  $C^*$  is the estimated drug concentration at 8 hours, and  $\lambda$  is the slope (on a log-concentration scale) of the terminal phase of the concentration-time curve, i.e., an exponentially decaying time course was assumed for our extrapolation to infinite time. The exponential amplitude and decay rate variables,  $C^*$  and  $\lambda$ , respectively, were estimated by regression over the last three data points.

**Assay for hepatic aldehyde oxidase activity.** Approximately 0.5 g of normal liver tissue was thawed in ice-cold PBS. The tissues were homogenized using a sonic dismembrator in a homogenization buffer, consisting of 50 mmol/L Tris-HCl (pH 7.5), 1 mmol/L EDTA, and 10% glycerol. The homogenate was centrifuged at  $12,000 \times g$  at  $4^\circ\text{C}$  for 60 minutes. The supernatant was recovered to a new tube, and stored at  $-80^\circ\text{C}$  until the enzyme assay was done. The protein concentration of the supernatant was determined by the method of Bradford (22). The reaction mixture contained 50 mmol/L Tris-HCl (pH 7.5), 1 mmol/L EDTA, 0.5 mmol/L IPdR, and 400  $\mu\text{g}$  protein of the crude enzyme in a final volume of 100  $\mu\text{L}$ . The mixture was incubated at  $37^\circ\text{C}$  for 30 minutes. The reaction was stopped by adding 300  $\mu\text{L}$  of ice-cold methanol. Twenty microliters of 100  $\mu\text{mol/L}$  bromodeoxyuridine was added as an internal standard. The mixture was centrifuged at  $12,000 \times g$  for 5 minutes to remove macromolecules. The supernatant was recovered and dried under reduced pressure, which was reconstituted in 100  $\mu\text{L}$  water and analyzed by HPLC. The HPLC apparatus and the running conditions were the same as the plasma nucleoside analogue analysis, described previously.

**Tumor regrowth assay.** Groups of 10 to 15 mice with a s.c. tumor xenograft were treated with IPdR (500 mg/kg q.o.d., 500 mg/kg q.d., or 166 mg/kg t.i.d.) or vehicle alone (control). On the IPdR treatment days 11 to 14 (for the q.d., t.i.d., and control groups) and days 25 to 29 (for the q.o.d. group), radiation therapy was delivered using 2 Gy / fraction  $\times$  4 days to the tumor xenografts. For irradiation, the mice were immobilized using a custom device without anesthesia and 6 MV photon beams were delivered to the tumors using a linear accelerator (Clinac 2100CD, Varian Medical Systems, Inc., Palo Alto, CA) with a 1.5 cm bolus. The adjacent normal tissues were shielded. Following irradiation, the tumor size (the maximum and its rectangular diameter) and the body weight were monitored daily. The tumor volumes were estimated by the formula:

$$R_1 \times R_2 \times R_2/2$$

where  $R_1$ , the maximum diameter, and  $R_2$ , the rectangular diameter.

The measurement of tumor regrowth was calculated by the time to grow up to 300% of the preirradiation volume. Tumor regrowth delay was quantified by the tumor regrowth of the irradiated groups (IPdR + IR and IR alone) minus that of the corresponding nonirradiated groups (IPdR alone and vehicle alone). The sensitizer enhancement ratio was defined as a ratio of  $T_{\text{IPdR+IR}}/T_{\text{IR alone}}$ , where  $T$ , tumor regrowth delay (days).

**Statistical methods.** Aldehyde oxidase activity was log-transformed for normalization before ANOVA analysis. IUdR incorporation was analyzed using a linear additive ANOVA model with day, dose, and treatment as factors. In all analyses, the residuals satisfied the underlying model normality assumptions.

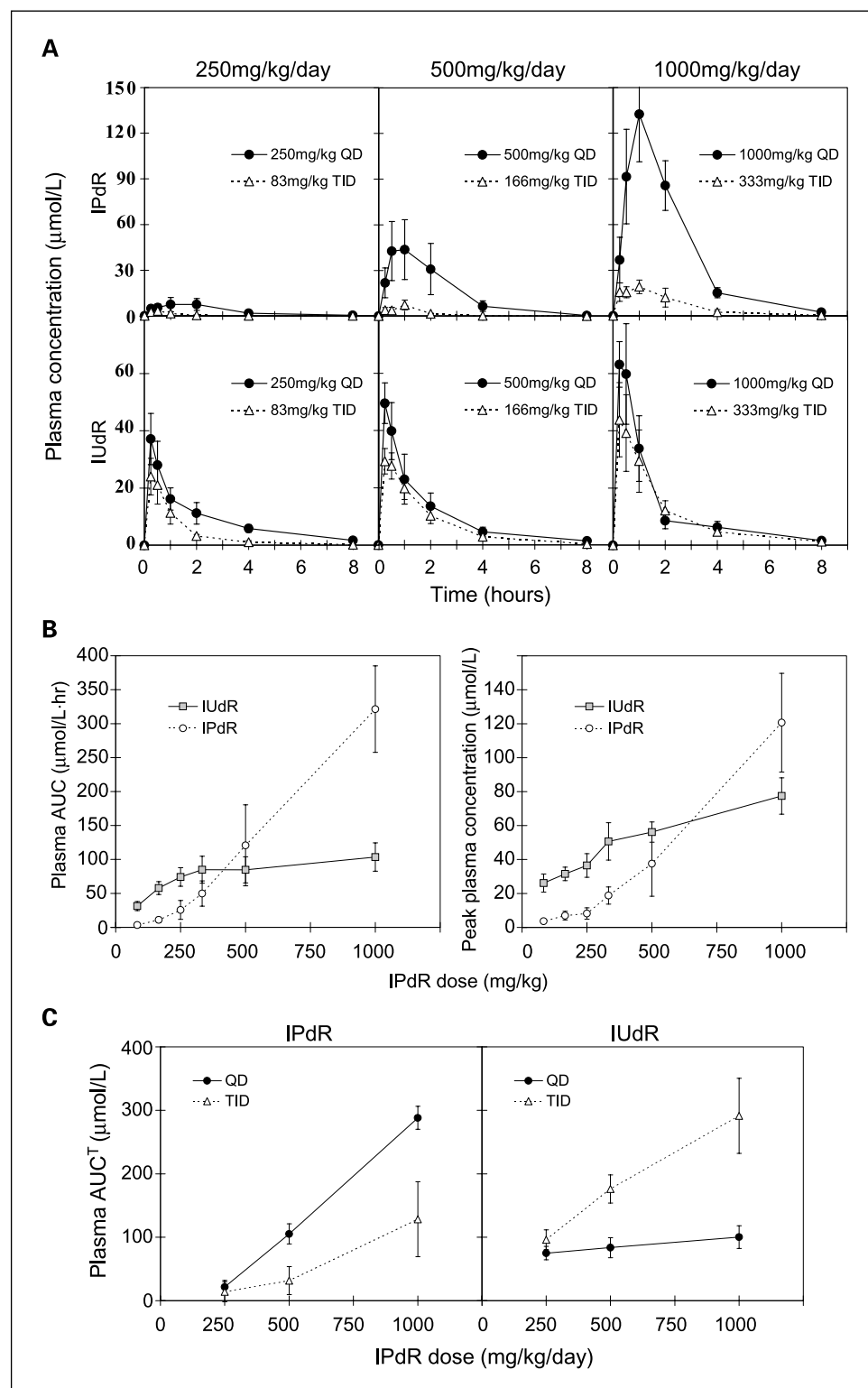
## Results

**Plasma pharmacokinetics using a q.d. versus t.i.d.  $\times$  14-day schedule.** Because we previously reported that the hepatic conversion of IPdR to IUdR may limit dose intensification (8, 16, 18), we questioned whether multiple IPdR doses divided daily results in a more effective conversion of IPdR to IUdR, and consequently higher IUdR-DNA incorporation. Athymic nude mice were given IPdR for 14 days at total doses of 250, 500, and 1,000 mg/kg/d using the q.d. and the t.i.d. schedules. Specifically, we compared the following 14-day treatment schedules: 250 mg/kg q.d. versus 83 mg/kg t.i.d.; 500 mg/kg q.d. versus 166 mg/kg t.i.d.; and 1,000 mg/kg q.d. versus 333 mg/kg t.i.d.

Figure 2A shows IPdR and IUdR plasma concentration-time curves following a single administration of IPdR (treatment day 1 of the six different dose schedules). The plasma IPdR concentration continued to increase and reached a peak at 1 hour after administration, whereas the peak of plasma IUdR concentration appeared at 15 minutes and declined in a biexponential fashion, suggesting rapid conversion of IPdR to IUdR as first-pass metabolism by hepatic aldehyde oxidase. AUCs and peak concentrations ( $C_{\text{max}}$ ) are shown as a function of given IPdR dose in Fig. 2B. IUdR AUCs reached a maximum level and then plateaued following a 333 mg/kg dose, whereas IPdR AUCs continued to increase in a dose-dependent manner. The IUdR  $C_{\text{max}}$  analysis showed a better dose-response compared with the IUdR AUC analysis, although the IPdR  $C_{\text{max}}$  curve showed a steeper increase than the IUdR  $C_{\text{max}}$  at doses  $\geq 333$  mg/kg. Hence, the enzymatic conversion from IPdR to IUdR was a rapid process, but saturable at  $\geq 333$  mg/kg. In order to compare the extent of IPdR conversion and subsequent IUdR exposure with the q.d. versus the t.i.d. schedule, the total daily AUC ( $\text{AUC}^T$ ) was defined as  $\text{AUC}_{0-\infty} \times 1$  for the q.d. schedule and  $\text{AUC}_{0-\infty} \times 3$  for the t.i.d. schedule (Fig. 2C). The  $\text{AUC}^T$  of IPdR and IUdR was dependent on IPdR dose ( $P < 0.001$  and  $P < 0.01$ ) and treatment schedule ( $P < 0.05$  and  $P < 0.01$ ). The  $\text{AUC}^T$  of IPdR and IUdR were similar using the q.d. versus the t.i.d. schedules at 250 mg/kg/d. However, if higher IPdR doses (500 and 1,000 mg/kg) were used, the t.i.d. schedule resulted in a higher IUdR  $\text{AUC}^T$  and lower IPdR  $\text{AUC}^T$  than the q.d. schedule, suggesting a saturation in enzymatic conversion of IPdR to IUdR using the higher IPdR doses and a more efficient conversion with the t.i.d. schedule.

We also analyzed plasma IPdR and IUdR pharmacokinetics using the q.d. versus the t.i.d. schedules (500 mg/kg/d) on the last day of the 14-day IPdR treatments (Fig. 3A). Compared with day 1, there was an increase of IPdR AUC, whereas IUdR AUC was considerably decreased. However, the t.i.d. schedule continued to provide significantly higher IUdR  $\text{AUC}^T$  than the q.d. schedule ( $P < 0.001$ ).

Hepatic aldehyde oxidase activity was measured *in vitro*. The activities on day 14 of the IPdR treatments were compared with the baseline activity of the control mice (Fig. 3B). Consistent



**Fig. 2.** A, plasma concentration-time curves of IPdR and IUdR following a single oral IPdR administration (treatment day 1). Athymic nude mice were treated with the three different total daily dosages of IPdR (250, 500, and 1,000 mg/kg). Two different treatment schedules were compared at each daily dosage; once daily (q.d.) versus three equally divided dosing a day with >6-hour intervals (t.i.d.); bars, SE of four to six animals. B, comparison of the AUCs and peak plasma concentrations of IPdR and IUdR, following a single oral IPdR administration (treatment day 1) as a function of IPdR dose. The IUdR AUCs reached a maximum level following a 333 mg/kg dose, whereas IPdR AUCs continued to increase in a dose-dependent manner. The IUdR  $C_{\text{max}}$  showed a better dose-response compared with the IUdR AUCs. However, the IPdR  $C_{\text{max}}$  curve showed a steeper increase than the IUdR  $C_{\text{max}}$  at doses  $\geq 333$  mg/kg. Points, mean; bars,  $\pm$  SE using four to six animals. C, comparison of the total daily AUC ( $\text{AUC}^T$ ) using the q.d. versus t.i.d. schedules. The  $\text{AUC}^T$  represents  $\text{AUC}_{0-\infty} \times 1$  for the q.d. schedule and  $\text{AUC}_{0-\infty} \times 3$  for the t.i.d. schedule. The  $\text{AUC}^T$  of IPdR and IUdR were dependent on IPdR dose ( $P < 0.001$  and  $P < 0.01$ ) and treatment schedule ( $P < 0.05$  and  $P < 0.01$ ). The t.i.d. schedule resulted in higher IUdR  $\text{AUC}^T$  and lower IPdR  $\text{AUC}^T$  than the q.d. schedule at the higher dosages (500 and 1,000 mg/kg/d); bars, SE of 5-10 animals.

with the plasma pharmacokinetic data, enzymatic conversion from IPdR to IUdR significantly decreased after the 14-day IPdR treatments in an IPdR dose-dependent ( $P = 0.01$ ) and treatment-dependent ( $P = 0.001$ ) manner (treatment-dose interactions suggested by Fig. 3B were not statistically significant). Dosing with  $\geq 250$  mg/kg of IPdR reduced the aldehyde

oxidase activity to  $\sim 40\%$  of the baseline level. The lower IPdR doses in the t.i.d. schedule (83 and 166 mg/kg) resulted in a smaller decrease in the enzyme activity.

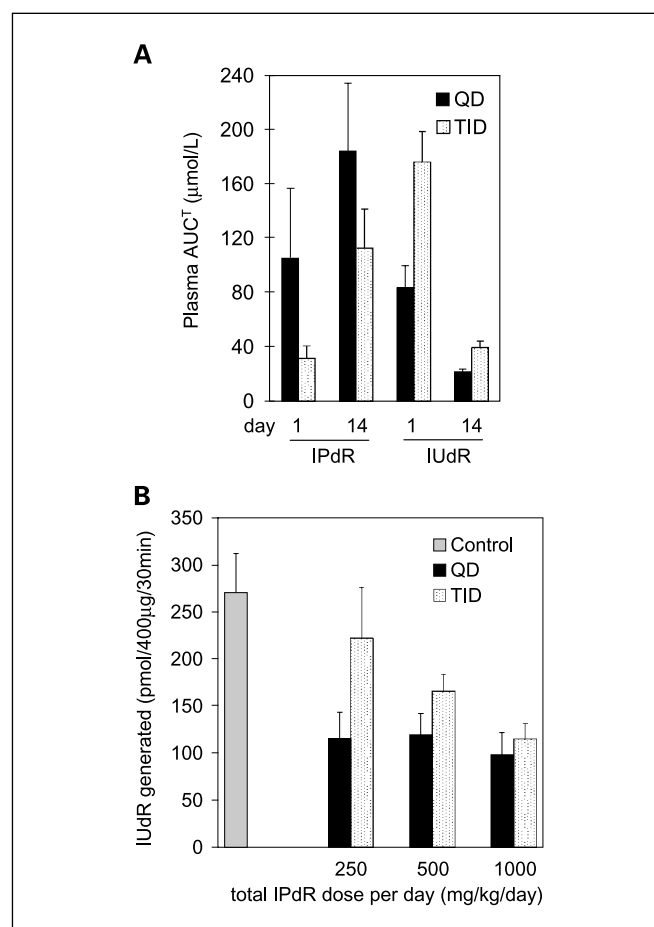
**Analysis of the percentage of 5-iodo-2'-deoxyuridine-DNA incorporation using a q.d. versus t.i.d.  $\times 14$ -day schedule.** The percentage of IUdR-DNA incorporation was measured in U251

xenografts as well as in small intestine and bone marrow, representing two normal proliferating tissues. Figure 4A shows the time courses of the percentage of IUdR-DNA incorporation during the 14-day IPdR treatments using 1,000 mg/kg q.d. versus 333 mg/kg t.i.d. Consistent with our data showing a higher IUdR AUC<sup>T</sup> using the t.i.d. schedule (Figs. 2C and 3A), the t.i.d. schedule showed a significantly higher IUdR incorporation than the q.d. schedule in tumor xenografts as well as normal proliferating tissues (bone marrow and small intestine) throughout the 14-day treatment period ( $P \ll 0.0001$  in all three situations). The percentage of IUdR-DNA incorporation increased significantly between days 3 and 14 in all three tissues ( $P < 0.0003$ ). Additionally, using three different total daily doses (1,000, 500, and 250 mg/kg/d), the percentage of IUdR-DNA incorporation on day 14 was compared using the q.d. versus the t.i.d. schedule (Fig. 4B). Significant dose dependence of percentage of IUdR-DNA incorporation was found in U251 xenografts ( $P = 0.02$ ) and small intestine ( $P < 0.0001$ ), but not in bone marrow. The higher incorporation using the t.i.d. schedule was evident in all three tissues; however, the increase in the percentage of IUdR-DNA incorporation using the t.i.d. schedule seemed to be dependent on the type of tissue.

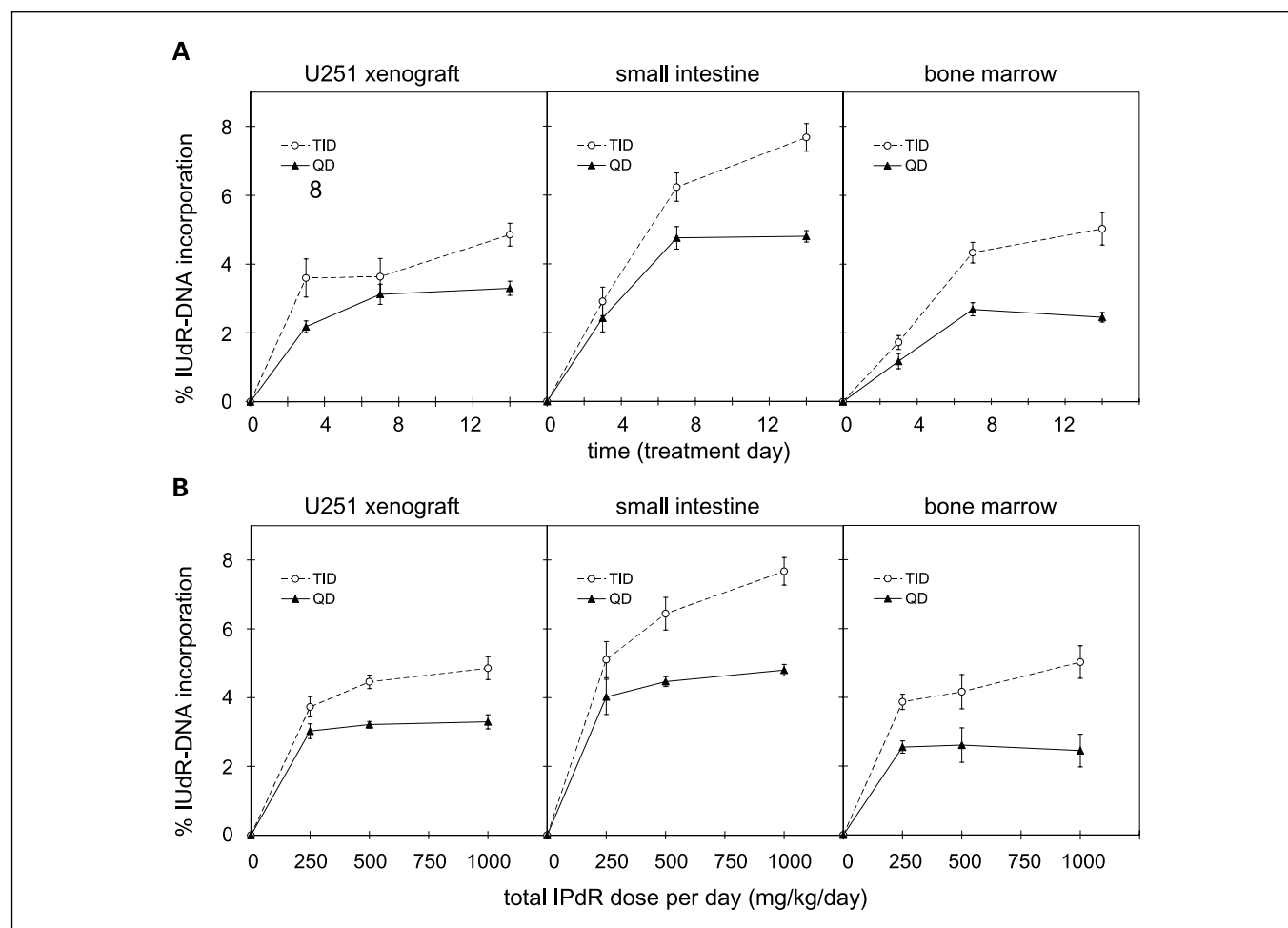
**Analyses of plasma pharmacokinetics and percentage of 5-iodo-2'-deoxyuridine-DNA incorporation following a fixed 5-iodo-2-pyrimidinone-2'-deoxyriboside (500 mg/kg) given q.d., q.o.d., and q.3.d.** We previously measured the activity of hepatic aldehyde oxidase serially following a single IPdR administration and found that hepatic aldehyde oxidase activity recovers within 2 to 3 days following a single IPdR dose of  $\leq 1,000$  mg/kg (18). Therefore, we questioned whether an increase in the time between IPdR dosings could result in more efficient conversion of IPdR to IUdR without hepatic aldehyde oxidase saturation. We were also interested in determining whether a longer treatment interval could affect the percentage of IUdR-DNA incorporation in tumor xenografts versus normal proliferating tissues. For this experiment, athymic nude mice were given 500 mg/kg of IPdR orally using the three different schedules: q.d. for 14 days (500 mg/kg  $\times$  14 doses); every other day (q.o.d.) for 23 days (500 mg/kg  $\times$  12 doses); and every 3rd day (q.3.d.) for 25 days (500 mg/kg  $\times$  9 doses). Plasma pharmacokinetics and IUdR-DNA incorporation were analyzed following these three treatment schedules. The plasma AUC<sub>0-8</sub> of IPdR and IUdR are shown in Fig. 5A. The longer IPdR administration intervals provide a greater IUdR AUC and a somewhat reduced IPdR AUC, suggesting that hepatic aldehyde oxidase activity partially recovers during the longer treatment intervals, although the IUdR AUC remained  $\sim 60\%$  of the single dose AUC at 500 mg/kg. However, the improved plasma pharmacokinetics with the longer dosing intervals did affect the percentage of IUdR-DNA incorporation. As shown in Fig. 5B, using the q.o.d. schedule, the percentage of IUdR-DNA incorporation in tumor xenografts was similar to that of the q.d. schedule, whereas the incorporation in the two normal proliferating tissues was significantly reduced. The q.3.d. schedule showed a decreased percentage of IUdR-DNA incorporation in tumor xenografts as well as the normal proliferating tissues, compared with the q.d. schedule.

**Tumor regrowth delay analysis following fractionated radiotherapy with the different 5-iodo-2-pyrimidinone-2'-deoxyriboside dosing schedules.** To test whether the difference in the

percentage of IUdR-DNA incorporation using the various IPdR dosing schedules could be translated to the extent of radiosensitization, we conducted a tumor regrowth assay using fractionated radiotherapy (2 Gy/d  $\times$  4 days; Fig. 6A). IPdR was given using 500 mg/kg q.o.d., 500 mg/kg q.d., or 166 mg/kg t.i.d. All IPdR treatment schedules showed significant increases of tumor regrowth delays compared with the control without IPdR (q.o.d., 29.4 days; q.d., 29.7 days; t.i.d., 34.7 days; radiotherapy alone, 15.7 days). Consistent with the analysis in the IUdR-DNA incorporation, the t.i.d. schedule showed the greatest regrowth delay, whereas the q.o.d. and the q.d. schedules resulted in a comparable effect on tumor regrowth delay. The extent of radiosensitization of each schedule was quantitated as a radiosensitizer enhancement ratio; q.o.d., 2.17; q.d., 2.20; and t.i.d., 2.56. There was no statistically significant difference between the q.o.d. and the q.d. schedules. The t.i.d. schedule showed significantly enhanced radiosensitization compared with the q.d. schedule ( $P < 0.05$ ).



**Fig. 3.** A, the AUC<sup>T</sup> was compared on treatment day 1 versus day 14 at IPdR dose of 500 mg/kg/d (500 mg/kg q.d. and 166 mg/kg t.i.d.). The AUC<sup>T</sup> of IPdR increased whereas the AUC<sup>T</sup> of IUdR substantially decreased on day 14 compared with day 1 in both the q.d. and the t.i.d. schedules, suggesting a reduction in enzymatic conversion following repetitive IPdR administrations. B, hepatic aldehyde oxidase activity following the 14-day IPdR treatment using the q.d. or t.i.d. schedules at the three different total daily dosages. The enzyme activity was measured *in vitro* at 8 hours after the last IPdR administration. Hepatic aldehyde oxidase activity was decreased in an IPdR dose-dependent ( $P = 0.01$ ) and treatment-dependent ( $P = 0.001$ ) manner; bars, SE of 5-10 animals.



**Fig. 4.** Comparison of the percentage of IUdR-DNA incorporation using the q.d. versus the t.i.d. schedules. *A*, time course of the percentage of IUdR-DNA incorporation using the q.d. and t.i.d. schedules at the total daily dosage of 1,000 mg/kg/d. *B*, dose-response of the percentage of IUdR-DNA incorporation following the 14-day IPdR treatments using the q.d. or t.i.d. schedules. The percentage of IUdR-DNA incorporation was significantly higher in the t.i.d. than the q.d. schedules ( $P \leq 0.0001$  in the all tissues). The two normal proliferating tissues showed more remarkable differences between the t.i.d. and the q.d. schedules than the tumor; bars, SE of 5-10 animals (day 0 values were assumed, rather than measured).

**Systemic toxicity and therapeutic index using the different 5-iodo-2-pyrimidinone-2'-deoxyribose dosing schedules.** Acute systemic toxicity was assessed by a change of body weight during the various IPdR treatment schedules (Fig. 6B). The t.i.d. schedule showed greater weight loss than the q.d. schedule, although both groups recovered to baseline levels within a week following the completion of the treatments. The q.o.d. schedule resulted in only mild temporary body weight loss in the initial period of the treatment, and did not show substantial weight changes thereafter. These effects on body weight loss seem to correlate with the greater percentage of IUdR-DNA incorporation in small intestine with the t.i.d. schedule and the lower incorporation using the q.o.d. schedule.

To compare therapeutic indices using the different IPdR treatment schedules, we analyzed the differences between the percentage of IUdR-DNA incorporation in tumor minus that in small intestine (tumor-intestine), and the percentage of IUdR-DNA incorporation in tumor minus that in bone marrow (tumor-marrow). Thus, greater positive values indicate a better therapeutic index. When (tumor-intestine) and (tumor-marrow) were fitted to an ANOVA model that included

treatment schedule (q.d. versus t.i.d.), day of treatment, and dose effects additively, the t.i.d. schedule yielded a significantly lower therapeutic index ( $P = 0.05$  and  $0.02$ ; IPdR dose dependence was not significant). Figure 6C shows (tumor-intestine) and (tumor-marrow) using the four different treatment schedules (all IPdR doses were combined in the q.d. and t.i.d. schedules). The (tumor-marrow) was greatest for the q.o.d. followed by the q.d. schedule ( $P < 0.05$  and  $P = 0.05$  when contrasted with q.3.d.). The (tumor-intestine) was greatest for the q.3.d., although the q.o.d. was not significantly different from the q.3.d. schedule. The t.i.d. and the q.d. schedules were significantly lower ( $P < 0.001$  and  $P < 0.01$  from the q.3.d.) for the (tumor-intestine). This suggests that the q.o.d. schedule may provide the best therapeutic index in this animal model.

## Discussion

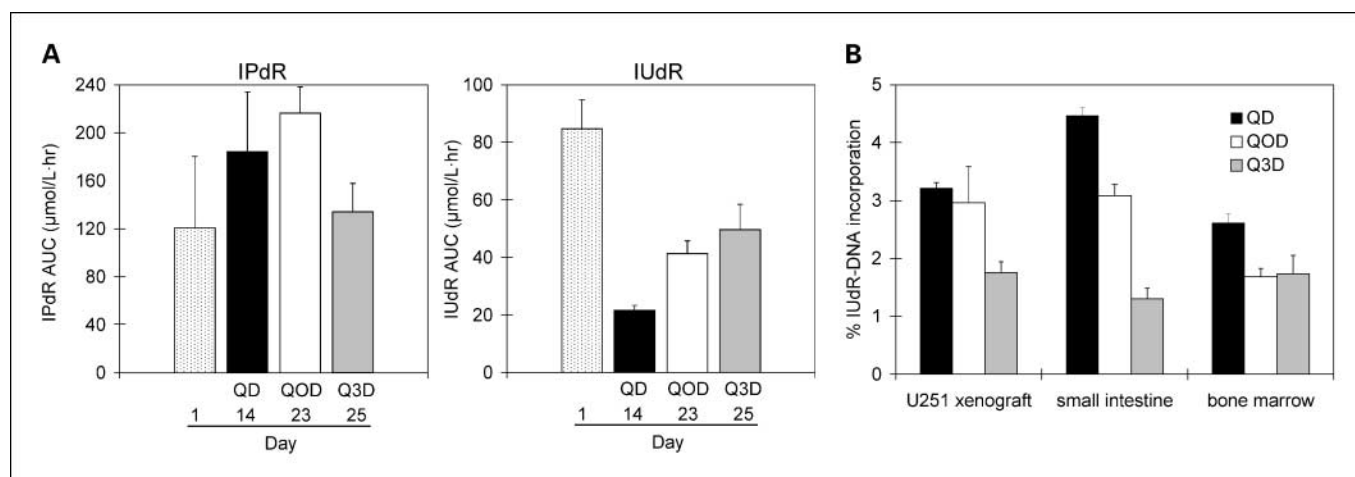
In this study, we tested a range of IPdR dosages and administration intervals, and found that the IPdR dosing schedule could affect IPdR pharmacokinetics and the percentage of

IUdR-DNA incorporation in human glioblastoma xenografts and mouse normal proliferating tissues. These changes in the pharmacologic variables subsequently affected tumor radiosensitization as well as systemic toxicity measured by a body weight change. With respect to IPdR pharmacokinetics, the alternative IPdR treatment schedules (t.i.d., q.o.d., and q.3.d.) improve the efficiency of enzymatic conversion of IPdR to IUdR compared with the q.d. schedule. Hepatic aldehyde oxidase in mice was saturated by IPdR at a single dose of  $\sim 333$  mg/kg (Fig. 2B). In addition, aldehyde oxidase activity was found to be significantly reduced during the various 14-day IPdR treatments (Fig. 3). The extent of decrease in aldehyde oxidase activity was dependent on administered IPdR doses as well as administration intervals. Based on an *in vitro* enzyme assay (Fig. 3B), a dose of 250 mg/kg results in a maximal reduction of hepatic aldehyde oxidase enzyme activity. In the t.i.d. schedule, the IPdR administration interval was decreased to  $\leq 8$  hours from 24 hours in the q.d. schedule. However, the shortened administration interval did not result in a substantial decrease in enzyme activity compared with the q.d. schedule. As a result, the t.i.d. schedule showed a higher IUdR AUC than the q.d. schedule, leading to higher percentage of IUdR-DNA incorporation in tumor xenografts and normal proliferating tissues (Fig. 4).

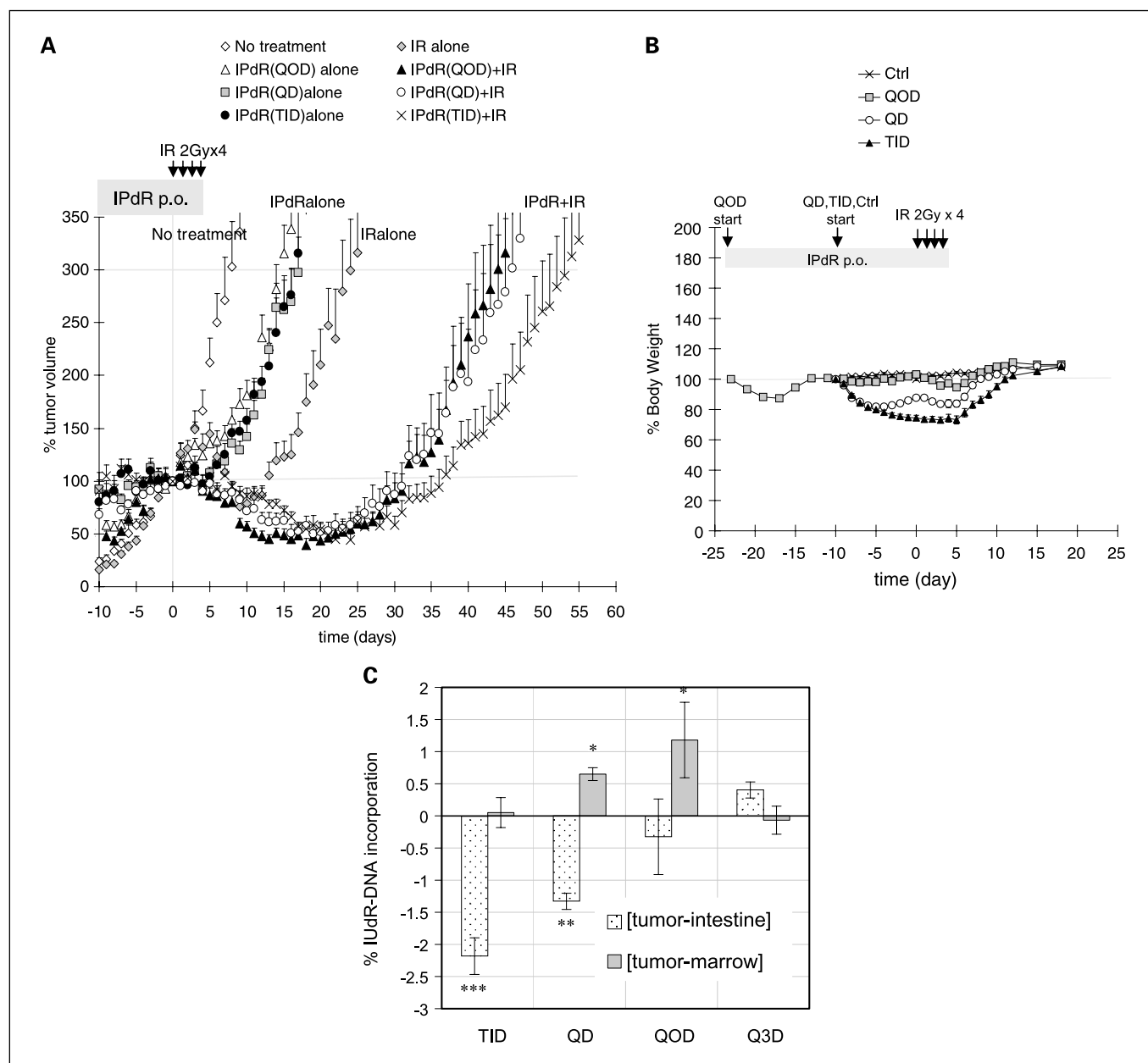
Previously, we found that aldehyde oxidase activity in mouse liver was decreased to  $\sim 50\%$  of the control at 24 hours but showed full recovery at 48 to 72 hours after single oral administration of IPdR (1,000 mg/kg; ref. 18). In the present study, we analyzed plasma IPdR and IUdR pharmacokinetics following a q.o.d. (day 23) or a q.3.d. treatment (day 25) schedule using a fixed dose (500 mg/kg). Consistent with our previous data using an *in vitro* enzyme assay, significantly improved conversion of IPdR to IUdR was found in both the q.o.d. and the q.3.d. schedules, although the recovery of enzyme activity was not complete. We have observed that excess amounts of plasma IPdR lead to reduced aldehyde oxidase activity following repetitive IPdR administrations. The precise mechanism of the down-regulation remains unclear,

and we can only speculate as to the mechanism involved here. We previously conducted a toxicology study using ferrets (16). No significant hepatic toxicities by IPdR were found in a histopathologic study and by analysis of serum liver function tests. Therefore, it is unlikely that the IPdR treatments caused a loss of enzyme activity due to direct hepatocellular toxicity. Other investigators reported that there is substrate inhibition of aldehyde oxidase from the liver cytosol of mouse (23), rat (24), and humans (25). Substrate inhibition is an inhibition of enzymes by excess substrate concentrations, and is one of the most common deviations from Michaelis-Menten kinetics, although the mechanism of this phenomenon is not completely understood (26). Because substrate inhibition is observed in nonphysiologic concentrations in most cases, the physiologic significance is not well established. However, the contribution of substrate inhibition to IPdR treatment cannot be excluded in this study using up to 1,000 mg/kg/d of IPdR where IUdR pharmacokinetics were saturated at 250 to 333 mg/kg/d. Another possibility is that a cosubstrate (e.g., molecular oxygen) is rate-limiting the reaction and the depletion of cosubstrate contributes to the reduction of the enzyme activity.

The efficient conversion of IPdR and the consequent higher plasma IUdR AUC led to a higher percentage of IUdR-DNA incorporation using the t.i.d. schedule than the q.d. schedule. Moreover, a comparable percentage of IUdR-DNA incorporation in tumor xenografts was observed using the q.o.d. schedule despite the longer IPdR administration interval compared with the q.d. schedule. The enhancement of IUdR-DNA incorporation using the t.i.d. schedule was large enough to be translated into significantly greater tumor radiosensitization (Fig. 6A). Therefore, the t.i.d. schedule may be considered more effective than the other treatment schedules. Of great interest, however, was the observation that the alternative IPdR administration schedules changed the ratio of the percentage of IUdR-DNA incorporation in tumor versus normal proliferating tissues, leading to different levels of systemic toxicity. The data presented in this study suggest



**Fig. 5.** Comparison of the plasma AUC and percentage of IUdR-DNA incorporation using the schedules with longer administration intervals (every other day, q.o.d.; every 3rd day, q.3.d.) compared with the q.d. schedule. **A**, IUdR AUC and IPdR AUC on the last day of the indicated IPdR treatments compared with the first day. The saturation of IPdR conversion to IUdR recovered partially, most likely due to the longer administration intervals. **B**, the q.o.d. schedule resulted in a decrease of the percentage of IUdR-DNA incorporation in the normal proliferating tissues although maintaining comparable incorporation in the tumor compared with the q.d. schedule; apparent differences are statistically significant ( $P = 0.01$  or less); bars, SE of 10 animals.



**Fig. 6.** *A*, tumor regrowth assay using fractionated radiotherapy 2 Gy/d × 4 days with or without the various IPdR treatment schedules (500 mg/kg q.o.d., 500 mg/kg q.d., or 166 mg/kg t.i.d.). All IPdR treatment schedules considerably enhanced tumor regrowth delay compared with ionizing radiation alone. The t.i.d. schedule showed significantly greater regrowth delay than the q.d. schedule whereas there was no significant difference between the q.d. and the q.o.d. schedules; bars, SE of 10 to 15 animals. *B*, acute toxicity during the IPdR treatments assessed by a body weight change. The degree of toxicity was dependent on the treatment schedules in the order of t.i.d. > q.o.d. > q.d. (most error bars are within plot marks; SE of 5-10 animals). *C*, analyses of therapeutic index as a function of the IPdR treatment schedules. The percentage of IUDR-DNA incorporation in tumor minus that in the two normal proliferating tissues [(tumor-intestine) and (tumor-marrow)] were assumed to represent the therapeutic index (all IPdR doses were combined in the q.d. and t.i.d. schedules). The (tumor-marrow) was greatest for the q.o.d. schedule followed by the q.d. schedule (\*,  $P < 0.05$  and \*,  $P = 0.05$  when contrasted with q.3.d.). The (tumor-intestine) was greatest for the q.3.d. schedule, although the q.o.d. schedule was not significantly different from the q.3.d. schedule. The t.i.d. and the q.d. schedules were significantly lower (\*\*,  $P < 0.001$  and \*\*,  $P < 0.01$  from the q.3.d.) for the (tumor-intestine) comparison.

that an increased frequency of IPdR dosing (t.i.d. versus q.d.) results in greater normal tissue toxicities, whereas less frequent administration (q.o.d. and q.3.d.) may provide a better therapeutic index.

We realize that the pharmacokinetics of IPdR may differ in humans compared with athymic mice. Indeed, in a recent study of interspecies and sex differences in hepatic aldehyde oxidase activity using IPdR and zebularine, another pyrimidinone

analogue, as substrates, similar kinetic values of pyrimidinone analogue metabolism were found in liver cytosol extracts from humans and Sprague-Dawley rats, regardless of sex (27). However, the  $V_{max}$  and  $K_m$  values differed significantly between male and female CD-1 mice (27). In our athymic mouse study, no sex differences were found in IPdR pharmacokinetics nor in IUDR-DNA incorporation using the different dosing schedules. Regardless, in all tested species, the principal IPdR metabolite

of liver cytosol incubation is IUdR (8, 16, 27). Although there are always limitations when one attempts to extrapolate from mice to humans, our study suggests that these alternative treatment schedules may provide additional benefits for IPdR-mediated human cancer radiosensitization. An initial phase I clinical trial of oral IPdR as a radiosensitizer will soon be

initiated using the q.d. schedule, with emphasis on the pharmacokinetics of IPdR and IUdR measured throughout the 28-day drug treatment. These alternative schedules will be considered for future clinical testing if the pharmacokinetic analysis of our initial q.d. IPdR suggests significant reduction of hepatic aldehyde oxidase activity.

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