Preclinical Toxicity and Efficacy Study of a 14-day Schedule of Oral 5-Iodo-2-pyrimidinone-2'-deoxyribose as a Prodrug for 5-Iodo-2'-deoxyuridine Radiosensitization in U251 Human Glioblastoma Xenografts1

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ABSTRACT

In anticipation of an initial clinical Phase I trial in patients with high-grade gliomas of p.o. administered 5-iodo-2-pyrimidinone-2'-deoxyribose (IPdR) given daily for 14 days as a prodrug for 5-iodo-2'-deoxyuridine (IUdR)-mediated tumor radiosensitization, we determined the systemic toxicities and the percentage IUdR-DNA incorporation in normal athymic mouse tissues and a human glioblastoma xenograft (U251) after this dosing schedule of IPdR. Using a tumor regrowth assay of s.c. U251 xenografts, we also compared radiosensitization with this IPdR-dosing schedule to radiation therapy (XRT) alone (2 Gy/day for 4 days) or to XRT after continuous infusion IUdR for 14 days at the maximum tolerated dose in mice (100 mg/kg/day).

Athymic mice with and without U251 s.c. xenografts tolerated 750 or 1500 mg/kg/day of p.o. IPdR (using gastric lavage) for 14 days without weight loss or activity level changes during treatment and for a 28-day posttreatment observation period. The percentage IUdR-DNA incorporation in U251 tumor cells was significantly higher after p.o. IPdR (750 and 1500 mg/kg/day) for 14 days (3.1 ± 0.2% and 3.7 ± 0.3%, respectively) than continuous infusion IUdR for 14 days (1.4 ± 0.1%). Compared to XRT alone, a significant sensitization enhancement ratio (SER) was found with the combination of p.o. IPdR (1500 mg/kg/d) + XRT (SER = 1.31; P = 0.05) but not for the combination of continuous infusion IUdR + XRT (SER = 1.07; P = 0.57) in the U251 xenograft model. Based on these data and our previously published data using shorter IPdR dosing schedules, which also demonstrate an improved therapeutic index for IPdR compared to IUdR, an initial clinical Phase I and pharmacokinetic study of p.o. IPdR daily for 14 days is being designed.

INTRODUCTION

The halogenated dThd3 analogues, BUdR and IUdR, are a class of pyrimidine analogues that have been recognized as potential radiosensitizing agents since the early 1960s (1, 2). Their cellular uptake and metabolism are dependent on the dThd salvage pathway where they are initially phosphorylated to the monophosphate derivative by the rate-limiting enzyme, TK (1). After sequential phosphorylation to triphosphates, they are then used in DNA replication, in competition with dTTP, by DNA polymerase. Indeed, DNA incorporation is a prerequisite for radiosensitization of human tumors by the halogenated dThd analogues, and the extent of radiosensitization correlates directly with the percentage dThd replacement in DNA (3, 4). The molecular mechanisms of radiosensitization are most likely related to the increased susceptibility of dThd analogue-substituted DNA to the generation of highly reactive uracil free radicals by IR, which may also damage unsubstituted complementary-strand DNA (5–7). Repair of IR damage may also be reduced by pre-IR exposure to these analogues (8, 9).

Over the last decade, there has been renewed interest in these halogenated dThd analogues as experimental radiosensitizers in selected cancer patient groups (1, 2). These analogues are rapidly metabolized in both rodents and humans, principally with cleavage of deoxyribose and subsequent dehalogenation by hepatic and extrahepatic metabolism, when given by bolus infusion with a plasma half-life of <5 min (1). Consequently, prolonged continuous or repeated intermittent infusions over

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3 The abbreviations used are: dThd, thymidine; IPdR, 5-iodo-2-pyrimidinone-2'-deoxyribose; IUdR, iododeoxyuridine; BUdR, bromodeoxyuridine; TK, thymidine kinase; XRT, radiation therapy; SER, sensitization enhancement ratio; IR, ionizing radiation; MTD, maximum tolerated dose; HPLC, high-performance liquid chromatography.
several weeks during irradiation are necessary, based on in vivo human tumor kinetics, to maximize the proportion of tumor cells that incorporate these analogues during the S phase (10, 11). Recent Phase I and II trials using prolonged continuous or repeated intermittent i.v. infusions of BUdR or IUdR before and during XRT have focused primarily on patients with high-grade brain tumors (1, 9, 12, 13). These clinically radioresistant tumors have a quite rapid proliferation and are surrounded by nonproliferating normal brain tissues that show little to no DNA incorporation of the dThd analogues (1, 12). As such, high-grade brain tumors are ideal targets for this approach to radiosensitization. The results of these recent Phase I/II clinical trials suggest an improved outcome compared to XRT alone in patients with anaplastic astrocytomas and possibly in patients with glioblastoma multiforme (12, 13). A therapeutic gain in clinical radiosensitization using these halogenated dThd analogues may also exist for other types of clinically radioresistant cancers, including locally advanced cervical cancer, head and neck cancers, unresectable hepatic metastases from colorectal cancers, and locally advanced sarcomas, based on the results of other recent Phase I/II clinical trials (14–19). However, systemic toxicity to rapidly proliferating normal tissues (principally bone marrow and intestine) can limit the duration and dose rate of the drug infusion and consequently may limit the extent of human tumor radiosensitization (14–19). Indeed, the use of high dose, short (96 h) intermittent i.v. infusions of BUdR can result in significant systemic myelosuppressive and dermatological toxicities as recently reported (20).

Various pharmacological approaches have been tried experimentally and clinically to improve the therapeutic gain of halogenated dThd analogue radiosensitization in poorly responsive human tumors. The use of selective intra-arterial infusions to increase tumor bed drug concentrations has been used clinically for primary brain tumors and hepatic metastases with a suggested modest improvement in the therapeutic gain (16, 19, 21). Experimentally, biochemical modulation of the key enzymes involved in dThd analogue metabolism (TK) or in the maintenance of cellular deoxyribonucleotide triphosphate pools (both thymidylate synthase and ribonucleotide reductase) have been studied using in vitro and in vivo human tumor systems (reviewed in Ref. 1). Biochemical modulation of thymidylate synthase has also been tried in clinical Phase I trials using concomitant continuous infusions of IUdR with either 5-fluorodeoxyuridine or folinic acid (leucovorin), but no improvements in the therapeutic gain were found (22, 23).

Another clinically relevant strategy to improve the therapeutic gain of halogenated dThd analogue radiosensitization in poorly responsive (clinically radioresistant) human tumors is to use a less systemically toxic halogenated analogue that can be metabolized in vivo to the active drug by tumor tissue or a normal tissue. The use of p.o. administered IPdR as a prodrug for IUdR-mediated tumor radiosensitization is an experimental approach under development by our group over the last 5 years (24, 25). The original strategy of development of this pyrimidine nucleoside for antiviral therapy by Lewandowski and Cheng (26) was based on the hypothesis that nucleosides without an amino group or oxygen at position 4 could be used as substrates by viral TK but not by mammalian cellular nucleoside kinases. However, these same investigators found an aldehyde (“IPdR”) oxidase, most concentrated in rodent and human liver, which efficiently converts IPdR to IUdR (27). In two recent publications, we have documented an improved therapeutic gain for in vivo human tumor xenograft radiosensitization using daily p.o. dosing of IPdR for 6 days compared to either p.o. or continuous infusion IUdR for similar time periods using MTD schedules of IUdR (24, 25). Using human colon cancer (HT-29 and HCT 116) s.c. xenografts in athymic mice, we reported ≥2-fold increases in percentage IUdR-DNA tumor cell incorporation and ≥2-fold decreases in percentage IUdR-DNA incorporation in proliferating normal tissues (bone marrow and intestine) for p.o. IPdR compared to either p.o. or continuous infusion IUdR. Additionally, pharmacokinetic analyses of p.o. IPdR shows efficient metabolism of IPdR to IUdR with peak plasma mouse levels within 15–30 min (25). Using cytotoxic extracts from normal human liver specimens, we also found rapid conversion of IPdR to IUdR, suggesting high IPdR aldehyde oxidase activity in normal human liver, which was not found in normal human intestine specimens or in human colorectal liver metastases. Finally, using a tumor regrowth assay to assess IR response, we found a 1.5-fold enhancement (time to regrow to 300% initial tumor volume) with IPdR (1000 mg/kg/day for 6 days) plus fractionated XRT (2 Gy/day for 4 days) in a human colon cancer xenograft (HT29) compared to fractionated XRT alone (25). No enhancement of XRT response was found with continuous infusion IUdR plus fractionated XRT in this human colon cancer xenograft.

In anticipation of an initial Phase I and pharmacokinetic clinical trial of p.o. IPdR given daily for 14 days during XRT in patients with high-grade gliomas, we designed this preclinical athymic mouse study to determine the systemic toxicities and the percentage IUdR-DNA incorporation in normal mouse tissues and a human glioblastoma xenograft (U251) after this proposed human dosing schedule of IPdR. We also compared the extent of radiosensitization using a tumor regrowth delay assay of U251 xenografts after oral IPdR (daily for 14 days) plus fractionated XRT (2 Gy/day for 4 days) during the last 4 days of IPdR dosing to XRT alone or continuous infusion IUdR for 14 days plus XRT (2 Gy/day for 4 days on days 11–14 of the IUdR infusion). We have previously characterized the in vitro radiation response of U251 cells and found these cells to be radioresistant (9), typical of the clinical radiation response of glioblastoma tumors (1, 2).

MATERIALS AND METHODS

Drugs and Chemicals. IPdR was synthesized and provided by SuperGen Pharmaceuticals, Inc. (San Ramon, CA). IPdR was dissolved in water and sterile-filtered. IUdR (Sigma Chemical Company, St. Louis, MO) was dissolved in NaOH and then diluted to the appropriate concentrations for continuous infusion. Nucleoside standards (BUdR, dThd), enzymes for DNA digestion, and other chemicals were obtained from Sigma.

Cell Culture. The U251 human glioblastoma cell line was obtained from the Division of Cancer Treatment Tumor Registry, National Cancer Institute, Frederick Research Facility (Frederick, MD). The glial properties of this cell line have previously been confirmed by electron microscopy, S-100 staining, and glial fibrillary acidic protein staining (9). The cells were...
maintained in α-MEM (Life Technologies, Inc., Grand Island, NY) and supplemented with 10% FCS, essential and nonessential amino acids, and t-glutamine and gentamicin (Life Technologies, Inc.) at 37°C in a humidified 5% CO₂ atmosphere. Cells were passaged weekly to maintain exponential growth. Cultures were routinely tested and found to be free of Mycoplasma contamination using a biological culture method (Mycoplasma Test Kit; Life Technologies, Inc.). The in vitro population doubling time of U251 cells was 26–28 h.

Tumor Xenograft Implantation. Exponentially growing U251 cells were detached from tissue culture plates using 0.1% trypsin in PBS-EDTA, suspended in α-MEM, and then resuspended in ice-cold PBS. The cells were then counted using an Elzone particle counter (Particle Data, Inc. Elmhurst, IL). Cells (2 × 10⁶) in 50 ml of PBS were injected s.c. into the caudal dorsal flank of 5–7-week-old female athymic nude mice. The animals were then housed under laminar flow with water and food available ad libitum. All mice were examined daily, and the s.c. tumor dimensions were measured three times weekly. Greater than 90% of s.c. implanted mice developed palpable tumors within 7 days of s.c. injection. Once the tumors reached a cross-sectional area of 0.25–0.30 cm², the daily p.o. administrations of IPdR (0, 750, or 1500 mg/kg/day for 14 days) or continuous infusion IUdR for 14 days at the previously established MTD (100 mg/kg/day) (25) were begun. For the IUdR continuous infusion, miniosmotic pumps (Alzet model 2001, Alza Corporation, Palo Alto, CA) were implanted s.c. under sterile conditions and general anesthesia (Pentobarbital, 55 mg/kg given i.p.) as previously published (10). At least two groups of six mice with s.c. U251 tumors were used for the assessment of systemic toxicity and measurements of the percentage IUdR-DNA incorporation into normal and tumor tissues at each IPdR dose level.

In Vivo Systemic Toxicity Studies and Mouse Tissue Sampling. Five-to-7-week-old female athymic nude mice (weighing 20–25 g; Harlan Sprague Dawley, Madison, WI), with and without U251 tumor xenografts transplanted to the s.c. tissues of the caudal dorsal flank, were housed under laminar flow ventilation with food and water and provided ad libitum. IPdR was administered p.o. using a gastric tube once daily at doses of 0, 750, or 1500 mg/kg/day for 14 consecutive days. The control group (0 IPdR dose) received a similar volume of sterile water by gastric tube each day. Weights were monitored daily along with visual inspection of mice to assess morbidity for 4 days before, during the 14-day treatment, and for 28 days after IPdR treatment. The appearance of the mice, including activity level, was recorded each day. Groups of six mice, with and without U251 tumor xenografts, were randomized to receive the three IPdR dose levels and were sacrificed 28 days after the IPdR treatment. Additionally, groups of six mice with U251 tumor xenografts received the 14-day IPdR treatment at the two dose (750 or 1500 mg/kg/day for 14 days) levels and were euthanized by cervical dislocation immediately after treatment to assess the percentage IUdR-DNA incorporation in normal (liver, intestine, and bone marrow) tissues and xenograft tumor tissue. After sacrifice, tissue samples of normal liver, intestine, and s.c. tumor were harvested and frozen in liquid nitrogen. Normal bone marrow was harvested from the femurs of mice by aspirating with α-MEM, washing twice with PBS, and pelleting by centrifugation (1000 × g for 10 min) before freezing in liquid nitrogen.

Digestion of DNA from Mouse Tissues. Approximately 500 mg of s.c. tumor, normal liver, and normal intestinal tissues were minced separately in Petri dishes containing 2.0 ml of ice-cold PBS. Bone marrow aspirates were similarly processed. Released cells were pipetted into a culture tube on ice. The Petri dish was then washed with 1 ml of PBS, and the cell suspensions of a particular tissue were combined. Cells were pelleted by centrifugation at 1000 × g at 4°C for 10 min. DNA was extracted and digested by the method of Belanger et al. (28). The digestion mixture was incubated overnight at 37°C. Four hundred-ml samples were ultrafiltered using Millipore ultrafree-MC units (Millipore, Bedford, MA).

HPLC Nucleoside Analysis of IUdR-DNA Incorporation in Xenograft Tumor and Normal Mouse Tissues. HPLC analysis of IUdR replacement of dThd in DNA was performed with the use of a Waters 600 E solvent delivery system on a 3.9 × 300 mm Bondapak reverse-phase column (Waters Corp. Milford, MA) according to the method of Belanger et al. (28). The mobile phase consisted of 100 mm of sodium acetate buffer (pH 5.45) plus 7% (v/v) acetonitrile (ACN). dThd and IUdR were detected at 290 mm. Peak identification and quantitation were performed against authentic nucleoside standards. The percentage IUdR-DNA incorporation was calculated as follows: ([(IUdR)/dThd] + IUdR) × 100.

Tumor Regrowth Assay. To assess the extent of in vivo p.o. IPD-related or continuous infusion IUdR-related radiosensitization in U251 s.c. xenografts, a standard regrowth delay assay was used. Mice with s.c. xenografts measuring 0.25–0.30 cm³ were randomized to one of seven treatment schedules including: control [no drug; no (sham) XRT]; XRT alone using 2 Gy/day for 4 days (days 1–4); XRT alone using 4 Gy/day for 4 days (days 1–4); p.o. IPD alone at 1500 mg/kg/day for 14 days; continuous infusion IUdR alone at 100 mg/kg/day for 14 days; p.o. IPD (1500 mg/kg/day for 14 days) with XRT delivered on days 11–14 using 2 Gy/day; and finally continuous infusion IUdR (100 mg/kg/day for 14 days) with XRT delivered on days 11–14 using 2 Gy/day. Groups of six mice were treated according to the seven treatment schedules, and the cross-sectional areas of the s.c. tumors were measured every other day during and after treatment for up to 60 days. The time to tumor growth to 300% of the initial tumor volume was used as an end point to measure the effect of treatment, similar to the tumor growth end point used in prior IPD studies (25).

For tumor irradiation, mice were restrained without sedation in lead jigs using a cutout to expose the s.c. tumor in the posterior third of the dorsal trunk. Irradiation was performed using a Philips RT 250 unit (Philips Medical Systems, Inc., Shelton, CT) operating at 250-kV potential and 15 mA using a 0.2-mm copper filter with a dose rate of 2.27 Gy/min. To improve dose homogeneity, s.c. tumors were irradiated using opposed fields with equal weighting. Sham-irradiated mice were handled in a similar fashion.

Statistical Analysis of Tumor Regrowth. To estimate the time at which 300% growth was achieved, we used a linear interpolation of the tumor growth data. Using that line, the day at which 300% growth was attained was computed. Our results were not sensitive to the number of points chosen for the linear interpolation of the tumor growth data. Using that line, the day at which 300% growth was attained was computed. Our results were not sensitive to the number of points chosen for the linear interpolation of the tumor growth data.

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regression. The time to 300% growth was compared among the seven treatment schedules using an ANOVA model, as previously described (25).

An SER was calculated as a simple ratio of the mean growth delay (time to 300% initial tumor volume) for IPdR or IUdR plus XRT (2 Gy/day for 4 days) to the mean growth delay for XRT alone (2 Gy/day for 4 days). To compute the SER, we used a natural logarithmic transformation of time to 300% growth. We then performed a two-sample t test on these logarithmic values. An advantage of using the logarithmic values is that we can derive confidence intervals directly for the SER. All analyses were performed in SAS using the generalized linear model procedure (29).

**Measurement of Hepatic IPdR Aldehyde Oxidase Activity in Athymic Mice.** Hepatic IPdR aldehyde oxidase activity was determined in 10 control athymic mice and in 15 treated athymic mice. Treatment consisted of a single p.o. dose of IPdR at 1000 mg/kg. Groups of three to five mice were then euthanized by cervical dislocation at days 1, 2, 3, and 4 after the single p.o. IPdR dose to determine the enzyme activity of hepatic IPdR aldehyde oxidase. The dose of 1000 mg/kg was selected based on our prior IPdR pharmacokinetic study in athymic mice showing prolonged IPdR plasma levels at 45–90 min after dosing with scissors. Samples were centrifuged at 10,000 × g for 20 min, the supernatant was removed to a fresh tube, and protein determinations were done by Bio-Rad assay. The reaction mixture contained 50 mM Tris-HCl (pH 7.5), 1 mM ATP-MgCl₂, and 10% glycerol was used after mincing with scissors. Samples were centrifuged at 10,000 × g for 20 min, the supernatant was removed to a fresh tube, and protein determinations were done by Bio-Rad assay. The reaction mixture contained 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.2 mM IPdR, and 10 μg of protein in a final volume of 100 μl. The mixture was incubated for 1 h at 37°C, and the reaction was stopped by adding 200 μl of ACN. The protein precipitate was removed by centrifugation at 5000 × g for 40 min. The supernatant was recovered and evaporated to dryness. Samples were resuspended in 100 μl of HPLC running buffer and 100 mM ammonium acetate (pH 6.8) containing 7% ACN. HPLC analyses of samples were performed on a Waters system as described previously. The absorbance of the eluate was monitored at 230 nm, with a flow rate of 2 ml/min. IPdR aldehyde oxidase activity in athymic mouse liver was expressed as the pmols of IUdR generated per h per 10 μg of protein.

**RESULTS**

The systemic toxicity of IPdR given as a daily p.o. bolus for 14 days at 750 and 1500 mg/kg/day is presented in Fig. 1. Using a change in the percentage body weight during drug treatment and for up to 28 days after treatment as an index of systemic toxicity, we found essentially no change in the percentage body weight gain for the 6 weeks of observation during and after drug treatment for the two IPdR groups compared to the control group receiving similar volumes of sterile water by gastric tube. Additionally, no other adverse effects were recorded regarding animal activity and behavior in the three groups by daily observation. As such, we did not reach an MTD of IPdR using this 14-day daily p.o. bolus schedule. No further dose escalation was used because of the increased risk of pulmonary aspiration with larger drug volumes (>2000 mg/kg/day) as we reported previously (25).

In Table 1, we present the percentage IUdR-DNA incorporation in cell extracts from U251 tumor tissue and from three normal tissues determined immediately after the 14-day treatment with p.o. IPdR. We found a modest increase in the percentage IUdR-DNA in both tumor (3.1 ± 0.2% and 3.7 ± 0.3%) as well as normal small bowel tissue (2.8 ± 0.1% and 3.3 ± 0.3%) as the dose of IPdR was increased from 750 to 1500.

![Fig. 1 Systemic toxicity as assessed by the percentage body weight change in athymic nude mice with and without s.c. U251 tumor xenografts after daily p.o. bolus administration of IPdR compared to control (sterile water lavage). The IPdR drug doses are illustrated on the figure. Data points, average percentage body weight at each day during treatment and for 28 days after treatment compared to controls (no drug; bars, SE; at least 12 animals were evaluated in each group).](clincancerres.aacrjournals.org)
mg/kg/day, respectively. In contrast, no significant increase in percentage IUdR-DNA incorporation was found in normal bone marrow (1.1 ± 0.1% and 1.2 ± 0.2%) and normal liver (0.2 ± 0.1% and 0.3 ± 0.1%) with the two IPdR dose levels, respectively. These changes in percentage IUdR-DNA in the two proliferating normal tissues (bone marrow and small bowel) are comparable to levels found in athymic mice exposed to p.o. IPdR doses of 1000–2000 mg/kg/day for 6 days as we previously reported (25). The percentage IUdR-DNA incorporation into normal bone marrow in ferrets after a 14-day IPdR treatment at 1500 mg/kg/day is also shown in Table 1, and it is quite similar to the mouse data. The low IUdR-DNA incorporation (<1%) found in normal mouse liver is reflective of its low proliferative activity, and the values are similar to our prior studies (24, 25). The percentage IUdR-DNA incorporation in U251 tumor cells was significantly higher after either 750 or 1500 mg/kg/day of p.o. IPdR for 14 days (3.1 ± 0.2% and 3.7 ± 0.3%, respectively) than continuous infusion IUdR for 14 days (1.4 ± 0.1%) at the MTD. This difference of ≥2-fold increase in percentage incorporation in this human glioblastoma tumor xenograft with p.o. IPdR compared to continuous infusion IUdR at the MTD is also comparable to our previous results using a shorter (6-day) drug infusion schedule in two different human colon cancer s.c. xenografts (HCT 116 and HT-29; Refs. 24 and 25).

In Fig. 2, we compare the effects on tumor growth following the six different treatments compared to a control (no treatment). The time in days (mean ± SE) to reach three times (300%) the pretreatment cross-sectional area (day 0) was used to measure the effect of the different treatments. Significant effects on tumor growth delay were found with both schedules of XRT alone (2 Gy/day for 4 days; 34.25 ± 3.24 days for 2 Gy/day for 4 days and 52.42 ± 3.14 days for 4 Gy/day for 4 days; *P*, 0.001) compared to untreated controls (20.77 ± 3.00 days). However, no effect on tumor growth delay was found with either drug treatment alone (20.24 ± 3.55 days for continuous infusion IUdR; *P* = 0.910 and 22.62 ± 3.24 days for p.o. IPdR; *P* = 0.678) compared to untreated controls. The combination of continuous infusion IUdR for 14 days and XRT (2 Gy/day for 4 days) on days 11–14 of IUdR resulted in a growth delay to 38.11 ± 3.01 days, which was significantly different from those of control (*P*, 0.001) but not from XRT alone (2 Gy/day for 4 days; *P* = 0.522). In contrast, the combination of p.o. IPdR for 14 days and XRT (2 Gy/day for 4 days on days 11–14 of p.o. IPdR) resulted in a

<table>
<thead>
<tr>
<th>Drug</th>
<th>Normal intestine</th>
<th>Normal bone marrow</th>
<th>Normal liver</th>
<th>U251 tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td>IPdR (750 mg/kg/day for 14 days)</td>
<td>2.8 ± 0.1%</td>
<td>1.1 ± 0.2%</td>
<td>0.2 ± 0.1%</td>
<td>3.1 ± 0.2%</td>
</tr>
<tr>
<td>IPdR (1500 mg/kg/day for 14 days)</td>
<td>3.3 ± 0.3%</td>
<td>1.2 ± 0.1% (1.9 ± 0.1%)*</td>
<td>0.3 ± 0.1%</td>
<td>3.7 ± 0.3%</td>
</tr>
<tr>
<td>IUdR (100 mg/kg/day for 14 days)</td>
<td>ND (≥8%)</td>
<td>ND (≥6%)*</td>
<td>ND (≥1%)*</td>
<td>1.4 ± 0.1%</td>
</tr>
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</table>

* Estimated percentage IUdR-DNA replacement from prior athymic mouse study with continuous infusion IUdR for 6 days (25).
* Measured percentage IUdR-DNA replacement using ferrets (T. Kinsella, unpublished data).
* ND, not done.
growth delay of 44.87 ± 3.24 days, which was significantly different from those of the control (P < 0.001), IPdR alone (P < 0.001), and XRT alone (P = 0.016). The SER defined as a simple ratio of the mean growth delay (time to 30% initial tumor volume) for IPdR and XRT (2 Gy/day for 4 days); XRT alone (2 Gy/day for 4 days) was 1.31, with a 95% confidence interval of 1.15–1.52 (P = 0.05). The SER for IUdR and XRT was only 1.07 with a 95% confidence interval of 0.92–1.30 (P = 0.57).

Finally, we measured the IPdR aldehyde oxidase activity in athymic mouse liver for up to 4 days after a single p.o. IPdR dose of 1000 mg/kg (Fig. 3). This analysis was prompted by a prior IPdR pharmacokinetic study in athymic mice where we found prolonged IPdR plasma levels for up to 90 min after single p.o. IPdR doses of ≥1000 mg/kg (25). The data suggested possible enzyme saturation. In the present study, we found IPdR aldehyde oxidase activity to be decreased to ~50% of normal at day 1 after drug administration but full recovery to normal by days 2–4 (Fig. 3).

**DISCUSSION**

In a previous in vitro study using U251 human glioblastoma xenografts in athymic mice, we concluded that the predominant mechanism of IUdR radiosensitization was related to an increased production of DNA damage by IUdR rather than an inhibition of IR-related DNA repair (9). We based this conclusion on studies evaluating in vitro clonogenic survival and IR-related repair half-times after acute (1.4 Gy/min) and chronic (0.2–0.8 Gy/h) radiation exposures in U251 cells with and without prior exposures to clinically relevant (2 μm) IUdR concentrations. No significant interactions were found between the radiation dose rate and prior IUdR exposures that resulted in percentage IUdR-DNA tumor cell incorporations of 4–8%. Additionally, IR repair half-times for both potentially lethal damage repair and sublethal damage repair were similar in the presence or absence of IUdR. Similar results were found for another human glioblastoma cell line, G18, in this in vitro study (9). We argued that these findings may have clinical relevance because the proposed mechanism would suggest that human tumors that incorporate IUdR, regardless of their radiation damage repair capacity, can be expected to demonstrate radiosensitization.

More recently, we reported that human tumor cells deficient in DNA mismatch repair, related to lack of expression of either the hMLH1 or hMSH2 proteins, show significantly greater IUdR-DNA incorporation and IUdR tumor radiosensitization than genetically matched DNA mismatch repair proficient cells (30). Indeed, some human high-grade brain tumors, which are clinically resistant to high doses of radiation alone as well as to alkylating and methylating drugs, have been recognized experimentally and histologically to have microsatellite instability and defects in DNA mismatch repair protein expression related to promoter methylation as a possible explanation for the observed drug and radiation resistance (31, 32). Thus, based on our in vitro studies in human brain tumor cell lines and mismatch repair deficient human tumor cells (9, 30) and the clinical results of some Phase I–II trials suggesting an improved survival after continuous or repeated i.v. infusions of IUdR and XRT compared to XRT alone in patients with anaplastic astrocytomas and possibly with glioblastoma multiforme (12, 13, 16, 33), we continue to pursue translational research efforts to further improve the therapeutic gain of IUdR radiosensitization in high-grade human brain tumors.

In this in vivo study using U251 human glioblastoma xenografts in athymic mice, we extend our preclinical evaluation of IPdR as an oral prodrug for IUdR using a daily 14-day drug schedule. We report that IPdR can be administered daily as a p.o. bolus at up to 1500 mg/kg/day for 14 days without significant systemic toxicity in athymic mice (Fig. 1). Using the percentage IUdR-DNA incorporation in normal mouse intestine and normal mouse bone marrow as surrogate indices to predict systemic normal tissue toxicities (as found in clinical Phase I studies of continuous IUdR infusions; Refs. 22, 23, and 34), we found a ≥2-fold reduction in percentage IUdR-DNA cellular incorporation in normal mouse tissues with IPdR at 750-1500 mg/kg/day for 14 days (Table 1) compared to our previously published results with continuous infusion IUdR for 6 days at the MTD (7.9 ± 0.68% for mouse intestine and 6.0 ± 0.68% for mouse bone marrow; Ref. 25). As determined by its low proliferative rate, the percentage IUdR-DNA incorporation in normal liver after IPdR for 14 days is very low (≤0.3%; Table 1), and this result is comparable to our prior mouse normal liver data (25) and prior human normal liver data with continuous infusions of IUdR (35). We also found an improvement in the therapeutic gain with p.o. IPdR for 14 days compared to continuous infusion IUdR for 14 days with a ≥2-fold increase in the percentage IUdR-DNA tumor cell incorporation in U251 tumor xenografts (Table 1). Furthermore, we demonstrated again that significant tumor radiosensitization, as defined in our U251 tumor regrowth assay, is possible with coadministration of p.o. IPdR before and during XRT (Fig. 2).

We hypothesize that the observed improved therapeutic index for tumor radiosensitization by p.o. IPdR compared to a continuous i.v. infusion of IUdR found in both a human colorectal tumor xenograft (25) and in a human glioblastoma tumor

![Fig. 3 Hepatic IPdR aldehyde oxidase activity determined in pooled cytosolic extracts from groups of control (0 mg/kg IPdR) or treated (1000 mg/kg IPdR) mice. Enzyme activity is based on the in vitro conversion of IPdR to IUdR after a 1-h incubation of liver cytosolic extracts with IPdR as described. N represents the number of athymic mice in each assay group.](cancercreres.aacrjournals.org)
xenograft in athymic mice described above results from three interrelated factors. First, p.o. IPdR has little systemic toxicity in rodents (Refs. 24, 25, and 27 and this study). Indeed, in this study we found no body weight loss in athymic mice receiving either 750 or 1500 mg/kg/day for 14 days and observed for 28 days after IPdR treatment (Fig. 1). We previously reported a 10% weight loss in athymic mice receiving 2000 mg/kg/day for 6 days (25) and noted a similar weight loss in a recently completed preclinical toxicology study in ferrets (a nonrodent species) at 1500 mg/kg/day for 14 days. For comparison, athymic mice tolerated a continuous infusion of IUdR at 50 mg/kg/day for 6 days with 10–15% weight loss but experienced ≥20% weight loss using 100 mg/kg/day for 6 days (25). Thus, although we have not established the maximum tolerable dose for p.o. IPdR given daily for 6–14 days in either rodent or mammalian species, we have noted a ≥10% weight loss with IPdR doses of 1500–2000 mg/kg/day for up to 14 days. These systemic toxicity data have resulted in a calculated starting dose of 85 mg/m²/day for 14 days (~1/10 dose of 1500 mg/kg/day in ferrets) in our proposed clinical Phase I trial.

Second, we hypothesize that the low toxicity profile of p.o. IPdR in athymic mice may result in part from its pharmacokinetic properties (24, 25). After a single p.o. administration of IPdR using 250–1500 mg/kg, we found a very efficient conversion of IPdR, the prodrug, to IUdR, the active metabolite, within 15–20 min, resulting in peak IUdR plasma levels of 40–75 μM and IUdR plasma levels persisting at >20 μM for up to 90 min (25). However, using IPdR at ≥1000 mg/kg, there appeared to be a saturation of hepatic aldehyde oxidase, resulting in prolonged and higher plasma levels of IPdR as well as the two inactive metabolites, 5-iodo-2-pyrimidine and 5-iodouracil (25). In this present study in athymic mice, we extend our preclinical pharmacokinetic analysis of p.o. IPdR to measure hepatic IPdR aldehyde oxidase activity for up to 4 days after a single gavage at 1000 mg/kg. We found that there was an ~50% decrease in enzyme activity at day 1, but full recovery of enzyme activity occurred at days 2–4 post gavage (Fig. 3). In a more recent study of p.o. IPdR at 1500 mg/kg/day for 14 days in ferrets, we found that hepatic IPdR aldehyde oxidase activity was reduced to 50% of normal at day 14. Collectively, these mouse and ferret data of IPdR pharmacology indicate the need for a careful human pharmacokinetic study of p.o. IPdR as part of the initial Phase I clinical trial. In our proposed Phase I trial, plasma levels of IPdR and IUdR will be determined at 15 min, 30 min, and 2 h after the first p.o. dose and at 30 min after the p.o. dose on days 2, 5, and 14. We have already confirmed that normal human liver has significant IPdR aldehyde oxidase activity (25). Human liver IPdR aldehyde oxidase activity is cytosolic, protein-dependent, cofactor-independent, and inhibited by low concentrations of menadione and isovanillin (a selective inhibitor or an analogue substrate for aldehyde oxidase, respectively) but not by allopurinol (a selective inhibitor for xanthine oxidase; Ref. 25).

Third, we also hypothesize that the improved therapeutic index of p.o. IPdR compared to continuous infusion IUdR results in part from the 10–100-fold lower aldehyde oxidase activity found in other normal mouse tissues, including intestine, bone marrow, lung, brain, and kidney (25, 27). It is well established from the Phase I and II clinical trials of continuous or prolonged intermittent i.v. infusions of IUdR and its related analogue BUdR that the systemic toxicities to the bone marrow (myelosuppression) and intestine (diarrhea) limit the duration and dose rate of a continuous infusion, which may also limit the extent of human tumor radiosensitization (14–20). In our studies of p.o. IPdR in athymic mice with or without human tumor xenografts, we consistently find marked reduction in percentage IUdR-DNA incorporation in normal bone marrow and to a lesser extent in normal small intestine compared to continuous infusion IUdR (Refs. 24 and 25; Table 1). These two normal mouse tissues also show low levels of aldehyde oxidase activity compared to normal liver (24). Additionally, we reported that human small intestine had significantly lower IPdR oxidase activity (≥10-fold reduction) compared to human liver and that human intestine enzyme activity was not inhibited by isovanillin or allopurinol and was stimulated by menadione (25). These results indicate that human (and possibly rodent) intestine cytosol has some IPdR aldehyde oxidase activity that may result in local drug activation to IUdR and possibly higher DNA incorporation in normal intestine compared to normal bone marrow (Refs. 24 and 25; Table 1). Accordingly, systemic toxicity to normal intestine may be the dose-limiting toxicity to p.o. IPdR in humans and will be carefully monitored in the proposed Phase I clinical trial. The activity of IPdR aldehyde oxidase in normal human bone marrow has not been determined, but it is very low in mice (24). We have recently found low percentage IUdR-DNA incorporation in normal ferret bone marrow after p.o. IPdR at 1500 mg/kg/day for 14 days (Table 1), and no myelosuppression was found for this mammalian species after this dose schedule. As part of our proposed Phase I clinical trial, we plan to determine the percentage IUdR-DNA incorporation in circulating granulocytes during and after (for up to 7–10 days) the 14-day IPdR treatment as well as after blood counts.

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Preclinical Toxicity and Efficacy Study of a 14-day Schedule of Oral 5-Iodo-2-pyrimidinone-2 ′-deoxyribose as a Prodrug for 5-Iodo-2 ′-deoxyuridine Radiosensitization in U251 Human Glioblastoma Xenografts


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