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Fluorodeoxyuridine Improves Imaging of Human Glioblastoma Xenografts with Radiolabeled Iododeoxyuridine[1]

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ABSTRACT

Use of radiolabeled nucleotides for tumor imaging is hampered by rapid *in vivo* degradation and low DNA-incorporation rates. We evaluated whether blocking of thymidine (dThd) synthesis by 5-fluoro-2′-deoxyuridine (FdUrd) could improve scintigraphy with radio-dThd analogues, such as 5-iodo-2′-deoxyuridine (IdUrd). We first show *in vitro* that coincubation with FdUrd substantially increased incorporation of [^{125}I]IdUrd and [^{3}H]dThd in the three tested human glioblastoma lines. Flow cytometry analysis showed that a short coincubation with FdUrd (1 h) produces a signal increase per labeled cell. We then measured biodistribution 24 h after i.v. injection of [^{125}I]IdUrd in nude mice s.c. xenografted with the three glioblastoma lines. Compared with animals given [^{125}I]IdUrd alone, i.v. preadminsitration for 1 h of 10 mg/kg FdUrd increased the uptake of [^{125}I]IdUrd in the three tumors 4.8–6.8-fold. Compatible with previous reports, there were no side effects in mice observed for 2 months after receiving such a treatment. The tumor uptake of [^{125}I]IdUrd was increased ≤13.6-fold when FdUrd preadministration was stepwise reduced to 1.1 mg/kg. Uptake increases remained lower (between 1.7- and 5.8-fold) in normal proliferating tissues (*i.e.*, bone marrow, spleen, and intestine) and negligible in quiescent tissues. DNA extraction showed that 72–80% of radioactivity in tumor and intestine was bound to DNA. Scintigraphy of xenografted mice was performed at different times after i.v. injection of 3.7 MBq [^{125}I]IdUrd. Tumor detection was significantly improved after FdUrd preadministration while still equivocal after 24 h in mice given [^{125}I]IdUrd alone. Furthermore, background activity could be greatly reduced by p.o. administration of $KClO_4$ in addition to potassium iodide. We conclude that FdUrd preadministration may improve positron or single photon emission tomography with cell division tracers, such as radio-IdUrd and possibly other dThd analogues.

INTRODUCTION

Despite the advent of technologies, such as magnetic resonance imaging and [^{18}F]fluorodeoxyglucose positron emission tomography, identification of brain tumor recurrence after surgery or radiotherapy often remains difficult. Recently, several studies have focused on the development of scintigraphy based on dThd[3] or dThd analogues labeled with radionuclides, such as [^{11}C]dThd (1), [^{18}F]FdUrd (2), [^{18}F]fluorothymidine (3), [^{76}Br]BrdUrd (4, 5), or [^{124}I]IdUrd (6). Among them, IdUrd is of great interest, because it can be instantaneously labeled with γ- and β^+-emitting radioisotopes of convenient half-life (7). Because the iodine atom has a van der Waal's radius similar to the 5-methyl group that it replaces on the pyrimidine ring of dThd, IdUrd can be incorporated and retained in DNA for the entire life of a cell (8). However, use of radio-IdUrd, similar to radio-dThd or analogues, is limited by a low incorporation rate in tumor and a short biological half-life attributable to rapid *in vivo* catabolism and dehalogenation (6, 9). Having observed that combination of radiolabeled with unlabeled IdUrd could bypass some of these limitations (10), we investigated here the potential of FdUrd, another nucleotide analogue, to increase DNA-incorporation of radio-IdUrd by inhibiting the *de novo* synthesis of dThd (11). FdUrd, after conversion to FdUMP, inhibits TS by acting as nonconvertible substrate. The dTTP pool is subsequently depleted, and IdUrd, after phosphorylation through the nucleotide salvage pathway, can replace dTTP in DNA. Different groups have evaluated this mechanism for therapeutic purposes and observed variable results in which an increase in DNA-incorporation of radio-IdUrd was not always correlated with an increase in cytotoxicity (12–18). In view of these divergent observations, we postulated that a short administration of FdUrd could increase the amount of [^{125}I]IdUrd incorporated per cell, without increasing the number of cells in early S phase, which incorporates [^{125}I]IdUrd. Thus, combined use of FdUrd should be possible for improving the signal intensity of radio-IdUrd or other dThd analogues for diagnostic purpose, without additive cytotoxicity. Therefore, we conducted *in vitro* and *in vivo* studies to assess the potential of FdUrd for increasing DNA uptake and improving the diagnostic potential of radio-IdUrd as a tumor proliferation marker. Three human glioblastoma cell lines were chosen with different *p53* expression. Mutations on the gene *p53* often occur in glioblastoma (19, 20) and might differently affect cell response to radiation-induced damage (21, 22). Glioblastoma are potential candidates for clinical use of radio-IdUrd (6, 23, 24), because the surrounding normal brain has a very low cell division activity. Our initial *in vitro* studies have been subsequently confirmed *in vivo* in nude mice s.c. xenografted with the same glioblastoma lines, suggesting that our observations might be relevant for clinical application.

MATERIALS AND METHODS

Reagents. Noncarrier-added sterile [^{125}I]IdUrd (specific activity 74 TBq/mmol, 2000 Ci/mmol) and [^{3}H]dThd (specific activity 2.5–3 TBq/mmol, 70–85 Ci/mmol) were purchased from Amersham International (Buckinghamshire, United Kingdom). Quality control of radiochemical purity was performed with TLC (Tracemaster 20; Berthold, Bad Wildbad, Germany) on polyethyleneimine cellulose F (Merck, Darmstadt, Germany) with a mobile phase of 0.2 M ammonium bicarbonate. In all experiments, [^{125}I]IdUrd represented ≥80% of overall iodine-125 activity. FdUrd, dThd, and unlabeled IdUrd were obtained from Fluka Chemie AG (Buchs, Switzerland). Stock solutions were prepared in distilled water at a concentration of 5 mM and stored at −80°C. The IdUrd solution was used immediately after powder dissolution in 0.1 μM NaOH and adjustment of pH to 7.6 with HCl.

Cell Lines and Culture. Three human glioblastoma cell lines were chosen for their differential *p53* expression: (*a*) U87, homozygous wild type; (*b*) U251, homozygous mutant (both from the American Type Culture Collection); and (*c*) LN229, heterozygous (from our division). Cell culture medium containing RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, L-glutamine (0.3 mg/liter), and penicillin-streptomycin (0.1 mg/ml; all from Life Technologies, Inc., Grand Island, NY) was changed twice a week. Cells were kept at 37°C in a humidified incubator with 5% CO_2 and main-

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[3] The abbreviations used are: dThd, thymidine; FdUrd, 5-fluoro-2′-deoxyuridine; IdUrd, 5-iodo-2′-deoxyuridine; PI, propidium iodide; TS, thymidylate synthase; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-X nick end labeling; BrdUrd, bromodeoxyuridine; %ID/g, percentage of injected dose/gram; KI, potassium iodide.

tained in exponential growth phase. Cell doubling times in culture flasks as measured by daily counting over 1 week were 25.3 ± 0.8 h for U251, 34.5 ± 0.6 h for LN229 (10), and 34.6 ± 2.8 h for U87.

***In Vitro* Binding Assay.** To assess the effect of FdUrd on [^{125}I]IdUrd or [^{3}H]dThd incorporation in DNA, cells in exponential growth were incubated for 4 h at 37°C in 1 ml of complete medium containing 18.5 KBq (0.5 μCi) of [^{125}I]IdUrd or [^{3}H]dThd and different concentrations of FdUrd. According to published protocol (10, 16), cells were washed once with complete medium and twice with PBS, incubated for 10 min with 200 μl of trypsin-EDTA 1x, resuspended in 200 μl of complete medium, and lysed with 400 μl of 1 M NaOH. The DNA was precipitated by centrifugation after the addition of 400 μl of 10% trichloroacetic acid. A half volume was separated as one-half supernatant, and the remaining half volume was considered as sediment (containing DNA) + one-half supernatant. Radioactivity was determined in a gamma counter (Cobra QC 5002; Packard) for [^{125}I]IdUrd and in a liquid scintillation analyzer (Tri-Carb 2500 TR; Packard) for [^{3}H]dThd after the addition of 10 ml of scintillation liquid (Ultima Gold; Packard) per sample.

Fluorescence Cell Sorter Analysis. Cells were analyzed by two-parameter flow cytometry measuring both DNA content by PI (Fluka Chemie AG) and labeling index of IdUrd by a cross-reacting anti-BrdUrd primary antibody (Becton Dickinson, San Jose, CA) targeted by a FITC-conjugated goat anti-mouse antibody (Fluka Chemie AG). Adherent cells were labeled with 10 μM IdUrd for 1 h. Cells were then washed, resuspended, and fixed in 70% cold ethanol for 30 min on ice. The DNA was denatured by incubating the cells in 2 N HCl/0.5% BSA for 30 min at 20°C. The acid was neutralized by resuspending the cells in 1 ml of 0.1 M sodium borate at pH = 8.5. Cells were then resuspended as aliquots of 10^6 cells in 1 ml of 0.5% Tween 20/0.5% BSA/PBS and incubated for 30 min at 20°C with 20 μl of anti-BrdUrd. After centrifugation, the pellet was resuspended in 50 μl of 0.5% Tween 20/BSA/PBS and incubated for another 30 min at 20°C with 1 μg of antimouse IgG conjugated with FITC. Cells were then resuspended in 1 ml of PBS containing 5 μg/ml PI. The samples were analyzed on a flow cytometer (FACScan; Becton Dickinson) with a laser excitation at 488 nm for PI and FITC fluorescence.

Mice. Male Swiss homozygous *nu/nu* nude mice, obtained from Iffa Credo (L'Abresle, France), were kept under specific pathogen-free conditions, which included a separate facility with laminar flow racks, filter-topped cages, and autoclaved food and bedding. All experiments in nude mice were performed according to Swiss legislation and approved by the official committee on surveillance of animal experiments.

Tumor Model. A suspension of 5×10^6 cells was inoculated s.c. in the right flank of mice aged 7–8 weeks. After 30 days, solid tumors appeared and were maintained by serial s.c. transplantations. For experiments, animals with transplanted tumors at least two passages distant from the initial grafting were assigned to the different experimental groups by selecting similar mean tumor sizes (~0.3–0.5 grams) per group.

Biodistribution Study. Starting at least 1 day before the experiment, mice were given drinking water containing potassium iodide (0.2 grams/liter) to block thyroid uptake of iodine-125. Each experiment consisted of a minimum of three mice per treated group. Stepwise varying concentrations of FdUrd (30–1.1 mg/kg total doses) were administered in two fractions of half the total dose each injected i.v. in 200 μl of saline 60 and 30 min before i.v. injection of 250 KBq (6.76 μCi) [^{125}I]IdUrd in 200 μl of saline. Alternatively, eight i.p. injections of FdUrd in 100 μl of saline were given between 240 and 30 min before [^{125}I]IdUrd injection. Control mice had i.p./i.v. injections of saline alone followed by [^{125}I]IdUrd injection. Mice were sacrificed by CO_2 inhalation 24 h after [^{125}I]IdUrd injection, except for 11 animals sacrificed 6 h after injection. Blood (~0.5–1 ml) was obtained from the vein cava. Tumor, normal tissues, and carcasses were dissected and weighted; stomach and intestine were emptied for analysis. Radioactivity was measured in a gamma counter (Cobra QC 5002; Packard). Data are expressed as the %ID/g ± 1 SD.

Biodistribution after Potassium Perchlorate Administration. Two groups of five mice were given KI (0.2 grams/liter) in drinking water for 24 h. $KClO_4$ (2 grams/liter) was additionally given to one group. Mice were injected i.v. with 600 KBq (16.22 μCi) iodine-131 dissolved from a therapy capsule (Mallinckrodt, Petteu, the Netherlands), and biodistribution was measured 4 h later.

DNA Extraction from Tumor and Intestine. After dissection, tumor and intestine were collected and incubated for 4 h at 55°C in 500 μl of Tris-EDTA buffer containing 10% SDS and 25 μl of proteinase K (20 mg/ml), purchased from Fluka Chemie AG. After digestion, DNA was extracted by centrifugation after the addition of 500 μl of phenol, chloroform, and isoamyl-ethanol (25:24:1). The supernatant was collected in 1.5 volumes of isopropanol and centrifuged to precipitate the DNA (25). Radioactivity in DNA and in other tissue fractions was analyzed with a gamma counter (Cobra QC 5002; Packard).

Histology and Autoradiography. Tumors and intestine were frozen in liquid nitrogen and stored at −80°C. Samples were cut in 5-μm cryosections and fixed in 4% paraformaldehyde at pH = 7.4 in PBS. The cryosections were colored with hematoxilin/eosin before being dehydrated by increasing concentrations of ethanol (30–100%) and xylole. The slides were photographed and exposed at −80°C for 1 week. X-ray films were then developed and scanned, and images were analyzed with the PhotoShop computing program.

***In Situ* Nick End Labeling Assay.** Tumor and intestine cryosections of 5 μm were fixed in cold methanol at −20°C for 5 min and washed two times in PBS for 5 min. Slides were incubated for 1 min in 0.1% buffer citrate at pH = 6 and washed again two times in PBS for 5 min. TUNEL assay was performed according to the manufacturer's instructions (Roche, Basel, Switzerland). Slides were then washed three times with PBS for 5 min, incubated for 1 min with PI (Fluka Chemie AG), and analyzed by fluorescence microscopy. Positive controls were obtained after DNase Q1 digestion, and negative controls were obtained after incubation with FITC-dUTP in the absence of the enzyme terminal deoxynucleotidyl transferase. The DNA fragmentation rate was quantified by superposition of PI and FITC fluorescence.

Animal Scintigraphy. Whole-body scintigraphy of mice anesthetized with a mixture of Ketasol (Gräub AG, Bern, Switzerland) and tribromoethanol (Merck) was performed with a gamma camera (Toshiba GCA 9300 UI) using a high-resolution, low-energy collimator (LEHR-parallel). Mice were scanned at different times after the injection of 3.7 MBq (100 μCi) [^{125}I]IdUrd. To precisely measure the distribution of [^{125}I]IdUrd, the mice were killed after final scintigraphy at 24 h, and biodistribution was performed as described. In addition to KI (0.2 grams/liter), $KClO_4$ (2 grams/liter) was added to drinking water for at least 24 h in the experiments after the first series of scintigraphy.

Statistical Analysis. The variables were expressed as proportions or means ± 1 SD, as appropriate. Statistical significance was determined using a bilateral *t* test of equal variance. Statistical significance is reported for $P < 0.05$.

RESULTS

***In Vitro* DNA-Incorporation of [^{125}I]IdUrd and [^{3}H]dThd in the Presence of FdUrd.** DNA-incorporation of [^{125}I]IdUrd in the presence of different concentrations of FdUrd was assessed on three glioblastoma cell lines. As described previously (16), DNA-incorporation of [^{125}I]IdUrd was most rapid and linear within the first 4 h, and thereafter, it slowed down leveling off by 10 h. Therefore, *in vitro* experiments were conducted with an incubation time of 4 h on cells in exponential growth. According to the cell doubling time of each line, the highest baseline incorporation of [^{125}I]IdUrd alone was observed *in vitro* in U251 cells (0.92 MBq/cell), whereas incorporation was about three times lower in LN229 and U87 cells (0.32 and 0.26 MBq/cell, respectively; results not shown). Coincubation with FdUrd increased DNA-incorporation of [^{125}I]IdUrd in a concentration-dependent manner, increasing varying between 16.7- and 26.2-fold at 10 μM (Fig. 1). A 4-h preincubation with FdUrd was less effective (data not shown). Significant increase in DNA-incorporation of the physiological nucleotide [^{3}H]dThd was also obtained by coincubation with FdUrd, increasing varying between 4- and 5.4-fold at 10 μM FdUrd (Fig. 2).

Cell Cycle Distribution after Short or Long Exposures to FdUrd. Double staining fluorescence-activated cell sorter analysis was conducted to investigate whether incorporation increase of [^{125}I]IdUrd and [^{3}H]dThd by FdUrd was related to cell cycle changes. Cell cycle phases were revealed by PI fluorescence, and IdUrd content was revealed by staining with a cross-reacting anti-BrdUrd antibody

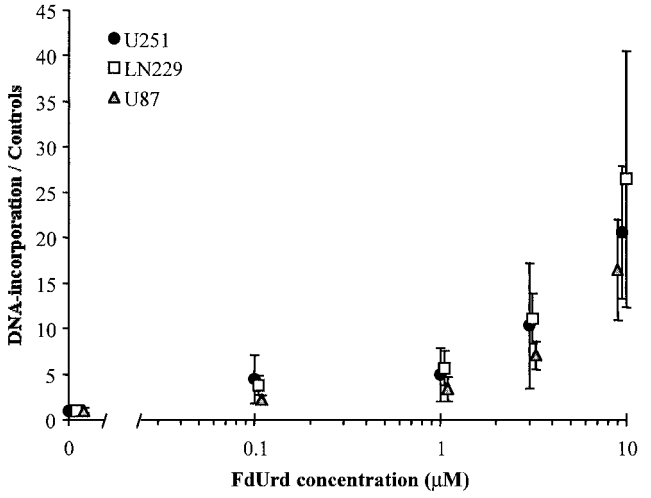

Fig. 1. DNA-incorporation of [^{125}I]IdUrd in the presence of FdUrd. Glioblastoma U251, LN229, and U87 were coincubated for 4 h with 18.5 KBq/ml [^{125}I]IdUrd and 0.1–10 μM FdUrd. The results represent the mean ±1 SD of at least three experiments per cell line, each consisting of three to four samples per tested condition. Results of each experiment have been normalized to a control group of three to four samples incubated for 4 h with [^{125}I]IdUrd alone.

revealed by fluorescinated antimouse IgG antibody. Compared with cells incubated for 1 h with 10 μM IdUrd alone (Fig. 3*A*), a 1-h coincubation with 10 μM FdUrd increased the signal of IdUrd detected per cell 3.6-fold (Intensity mean: 1240; Fig. 3*B*), without increasing the number of labeled cells in S phase. When using a 3-h preincubation with 10 μM FdUrd followed by a 1-h coincubation with 10 μM IdUrd and FdUrd, both the percentage of cells labeled with IdUrd (>37%) and the intensity of staining (Intensity mean: 633) increased (Fig. 3*C*). Antibody staining was negative for cells incubated with FdUrd alone or in the absence of primary antibody.

Biodistribution of [^{125}I]IdUrd in Xenografted Mice after FdUrd PreInjection. Compared with control mice receiving only the i.v. injection of [^{125}I]IdUrd, two i.v. preinjections of 5 mg/kg FdUrd (10 mg/kg total dose) given 60 and 30 min before [^{125}I]IdUrd increased the tumor uptake of [^{125}I]IdUrd from 4.8- to 6.8-fold in the three glioblastoma lines (Table 1). A longer pretreatment of 4 h gave similar results (data not shown). Different amounts of FdUrd (1.1–30 mg/kg total doses) were evaluated in one glioblastoma line. All tested doses produced significant tumor uptake increase of [^{125}I]IdUrd, but interestingly, the incorporation rate of [^{125}I]IdUrd tended to be higher (12.1- to 13.6-fold) at the lower concentrations of FdUrd (3.3–1.1 mg/kg; Table 2). The uptake increase of [^{125}I]IdUrd after FdUrd was higher in tumor than in normal tissues with rapid cellular turnover, leading to slightly more favorable tumor:normal tissues ratios (Table 2). In fact, the incorporation of [^{125}I]IdUrd in spleen, bone marrow, or intestine was increased between 1.7- and 5.8-fold after preadministration of FdUrd at the different doses, whereas the concentration of radioactivity in other slowly proliferating normal tissues, like liver or lung, remained negligible (Fig. 4).

Percentage of Total Tissue [^{125}I]IdUrd Radioactivity Incorporated in DNA. DNA-incorporation of [^{125}I]IdUrd was determined as the percentage of total radioactivity in intestine and tumor at 24 h after injection. The percentage of radioactivity associated with DNA after purification was similar for tumor and intestine. Furthermore, DNA-incorporation rates did not change significantly after preadministration of 3.3 mg/kg FdUrd, indicating a specific effect of FdUrd on [^{125}I]IdUrd incorporation (Table 3).

Cytotoxicity of Combined Administration of [^{125}I]IdUrd and FdUrd. Different experiments were conducted to evaluate the cytotoxicity of a combined administration of 250 KBq [^{125}I]IdUrd and 3.3 mg/kg FdUrd on tumor and normal rapidly dividing tissues represented by intestine. Samples were removed from mice sacrificed 24 h after [^{125}I]IdUrd injection. Apart from an overall more abundant radioactivity, tissue sections stained by hematoxilin/eosin and X-ray autoradiographies showed an unmodified histology and distribution pattern of radioactivity in tumor and intestine of mice preinjected with FdUrd *versus* mice injected with [^{125}I]IdUrd alone (data not shown). TUNEL assays were performed to compare the level of apoptosis between tumor and intestine in mice injected with [^{125}I]IdUrd alone or together with FdUrd. In two experiments, LN229 tumors of mice injected with [^{125}I]IdUrd + FdUrd showed a higher ratio of DNA fragmentation compared with tumors of mice injected with [^{125}I]IdUrd alone, whereas the ratio of DNA fragmentation in intestine did not increase after preinjections of FdUrd (data not shown). However, because apoptosis in normal intestine was already high in mice given [^{125}I]IdUrd alone, a minor additive cytotoxicity by FdUrd could have been masked. Finally, no side effects such as weight loss or diarrhea could be detected in three mice observed 2 months after administration of [^{125}I]IdUrd and 10 mg/kg FdUrd.

Contrast-enhanced Scintigraphy after FdUrd and Potassium Perchlorate. Scintigraphy was performed on anesthetized mice bearing glioblastoma xenografts at different times after the injection of 3.7 MBq [^{125}I]IdUrd. Tumor detection was already possible 4 h after [^{125}I]IdUrd injection in mice pretreated with FdUrd (data not shown), whereas tumors of similar size were barely visualized after 24 h in animals given [^{125}I]IdUrd alone (Fig. 5*A*). Comparison between Fig. 5, *A* and *B* shows that preadministration of 3.3 mg/kg FdUrd markedly increased the tumor visualization by [^{125}I]IdUrd at 24 h. However, despite administration of KI (0.2 grams/liter of drinking water for 24 h), a high background activity was detected, especially in the stomach (Fig. 5*B*). Stomach activity was shown by direct measurement in a gamma counter to be mainly concentrated in the stomach content with low activity in tissue. Therefore, the potential of $KClO_4$ to block free radiolabeled iodine secretion in the stomach was evaluated in biodistribution studies 4 h after i.v. injection of 600 KBq iodine-131 in two groups of five mice pretreated for 24 h, either with KI alone (0.2 grams/liter) or KI + $KClO_4$ (2 grams/liter) in drinking water. A significant reduction ($P = 0.03$) of 5.1 ± 3.6 to 0.8 ± 0.6%ID/g was observed in the stomach and intestine (including digestive content) of mice given $KClO_4$. Simultaneously, diminution

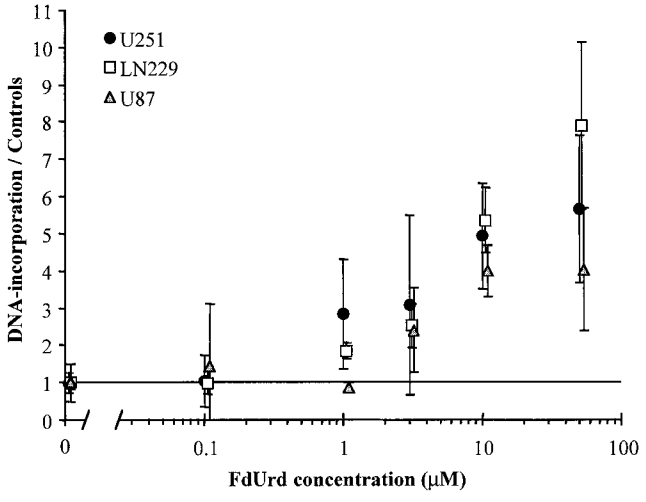

Fig. 2. DNA-incorporation of [^{3}H]dThd in the presence of FdUrd. Glioblastoma U251, LN229, and U87 were coincubated for 4 h with 18.5 KBq/ml [^{3}H]dThd and 0.1–50 μM FdUrd. The results represent the mean ±1 SD of three samples per tested condition, which have been normalized to a control group of three samples incubated for 4 h with [^{125}I]IdUrd alone.

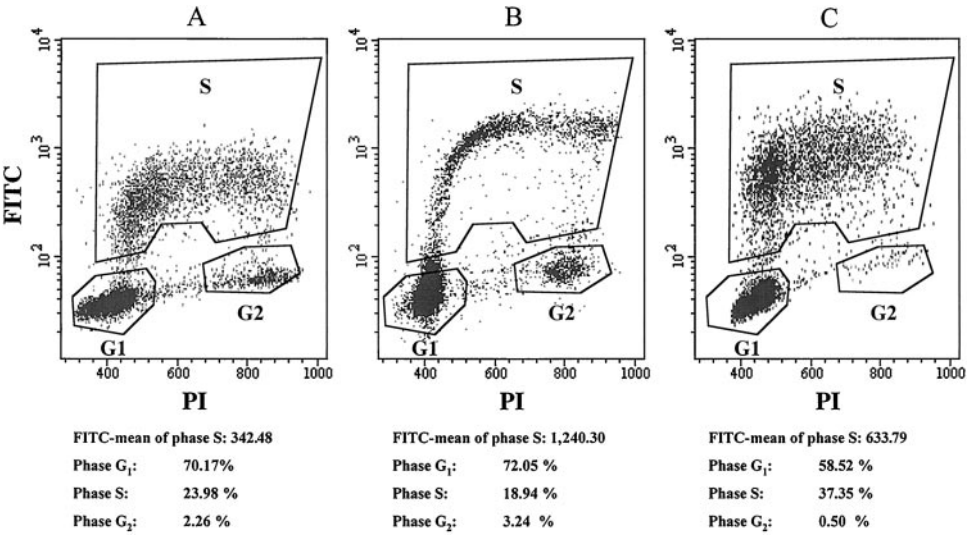

Fig. 3. Cell cycle distribution (*Phases G_1, S,* and G_2) and IdUrd incorporation rate after exposure of LN229 cells to FdUrd. *A,* incubation for 1 h with 10 μM IdUrd. *B,* coincubation for 1 h with 10 μM IdUrd and 10 μM FdUrd. *C,* incubation for 4 h with 10 μM FdUrd and addition of 10 μM IdUrd for the last hour. *Y* axis, rate of incorporation of IdUrd revealed by FITC fluorescence; *X* axis, cell cycle distribution revealed by PI fluorescence. Antibody staining with FITC was negative for cells incubated with FdUrd alone.

Table 1 *Tumor uptake of [^{125}I]IdUrd in mice xenografted with U87, LN229, or U251*

	U87		LN229		U251	
	%ID/g[a]	Nb[b]	%ID/g	Nb	%ID/g	Nb
[^{125}I]IdUrd alone	0.17 ± 0.07	7	0.17 ± 0.07	10	0.18 ± 0.04	8
[^{125}I]IdUrd + 10 mg/kg FdUrd	0.82 ± 0.30[c]	5	1.15 ± 0.36[c]	5	1.07 ± 0.40[d]	4
	4.8×[e]		6.8×		5.9×	

[a] %ID/g, percentage of injected dose per gram of tumor ± 1 SD.
[b] Nb, number of mice.
[c] $P < 0.001$.
[d] $P < 0.01$.
[e] Improvement ratio with FdUrd compared with mice receiving [^{125}I]IdUrd alone.

from 15.9 ± 11.1 to 6.2 ± 6%ID/g was observed in total body of mice given $KClO_4$ ($P = 0.12$). Radioactivity in the thyroid was always lower than 0.4%. Thus, in the next otherwise identical scintigraphy, mice were additionally pretreated with $KClO_4$ (2 grams/liter of drinking water for 24 h) before [^{125}I]IdUrd injection. Fig. 5*D* shows that tumor visualization by [^{125}I]IdUrd was drastically enhanced in mice given $KClO_4$ and FdUrd preadministration (3.3 mg/kg), with much better contrast and less background in normal tissues. The comparative scintigraphy was reproduced three times in different mice with similar results.

DISCUSSION

Glioblastoma are malignancies that have defied treatment. Part of the problem might be attributable to limitations of current diagnostic techniques, such as computerized tomography and magnetic resonance imaging, in localizing small collections of neoplastic glia within edematous or fibrotic parenchyma after therapy (23). Among alternative diagnostic procedures, use of radio-IdUrd would allow direct visualization and measurement of cell division. However, tumor visualization with radio-IdUrd encounters limitations attributable to low incorporation rate and high background radioactivity resulting from rapid *in vivo* degradation (6).

In the present study, we investigated the potential of FdUrd to improve tumor imaging with radio-IdUrd by favoring its incorporation into DNA after specific inhibition of *de novo* dThd synthesis (26). As reported previously for human glioma-derived cell lines (15), the ability to incorporate [^{125}I]IdUrd *in vitro* was low and varied between the three lines tested. However, coincubation with FdUrd greatly increased incorporation of [^{125}I]IdUrd into DNA of all cell lines. Similar biomodulation has been observed previously after exposure to FdUrd (14–16) and to other TS inhibitors, such as folinic acid in tumors of different origins, including breast and colon cancers, and leukemia of mouse and human origins (27).

As recently suggested (18, 28), an increased incorporation could reflect two distinct mechanisms: (*a*) an increase in the absolute uptake of IdUrd per cell; or (*b*) an increased number of cells in early S phase labeled with IdUrd. Our flow cytometry evaluation of IdUrd labeling index and cell cycle distribution indicated that a short exposure (1 h) to FdUrd increases the amount of IdUrd incorporated per cell, rather than the number of cells labeled with IdUrd. Thus, cytotoxicity with a short exposure to FdUrd should be restricted to the few cells that are naturally in S phase during treatment, whereas a longer exposure might extend toxicity to a larger cell population. Furthermore, other published results suggest that inhibition of DNA synthesis by FdUrd is transient, because recovery of dTTP pools after long exposures (6–24 h) to fluoropyrimidines has been observed (29), as well as reversion of FdUrd growth inhibition by unlabeled IdUrd and BrdUrd (30).

These *in vitro* observations could explain why i.p. injections of a total dose of 25 mg/kg FdUrd for 7 days led to a negligible weight loss of 0.1 grams in mice (11) and why i.v. injections of ≤210 mg FdUrd over 30 min was tolerated in rats (31). Moreover, rapid *in vivo* deglycosylation and dehalogenation, to which halogenated pyrimidines are subjected, probably limit toxicity to a large extent in the case of short exposure to FdUrd (32). In our *in vivo* studies, administration of total doses ranging from 1.1 to 30 mg/kg FdUrd was well tolerated in nude mice grafted with the three human glioblastoma lines. Histology, autoradiography, and TUNEL assays failed to demonstrate any additive cytotoxicity of FdUrd with [^{125}I]IdUrd in intestine, although a minor cytotoxicity of FdUrd might have been masked by

Table 2 *Modulation of [^{125}I]IdUrd uptake by FdUrd in mice xenografted with LN229*

FdUrd (mg/kg)	%ID/g of tumor[a]	%ID/g of small bowel[b]	Nb[c]
0	0.17 ± 0.07	0.65 ± 0.24	10
1.1	2.31 ± 0.76[d] (13.6×)[e]	2.74 ± 0.93[d] (4.2×)	4
1.7	2.24 ± 0.86[d] (13.2×)	1.92 ± 0.85[d] (3.0×)	5
3.3	2.05 ± 1.30[d] (12.1×)	1.72 ± 0.39[d] (2.6×)	11
10	1.15 ± 0.36[d] (6.8×)	1.36 ± 0.26[d] (2.1×)	5
30	1.12 ± 0.14[d] (6.6×)	2.02 ± 0.66[d] (3.1×)	4

[a] %ID/g, percentage of injected dose per gram of tumor ± 1 SD.
[b] %ID/g, percentage of injected dose per gram of small bowel ± 1 SD.
[c] Nb, number of mice.
[d] $P < 0.001$.
[e] Improvement ratio with FdUrd compared with mice receiving [^{125}I]IdUrd alone.

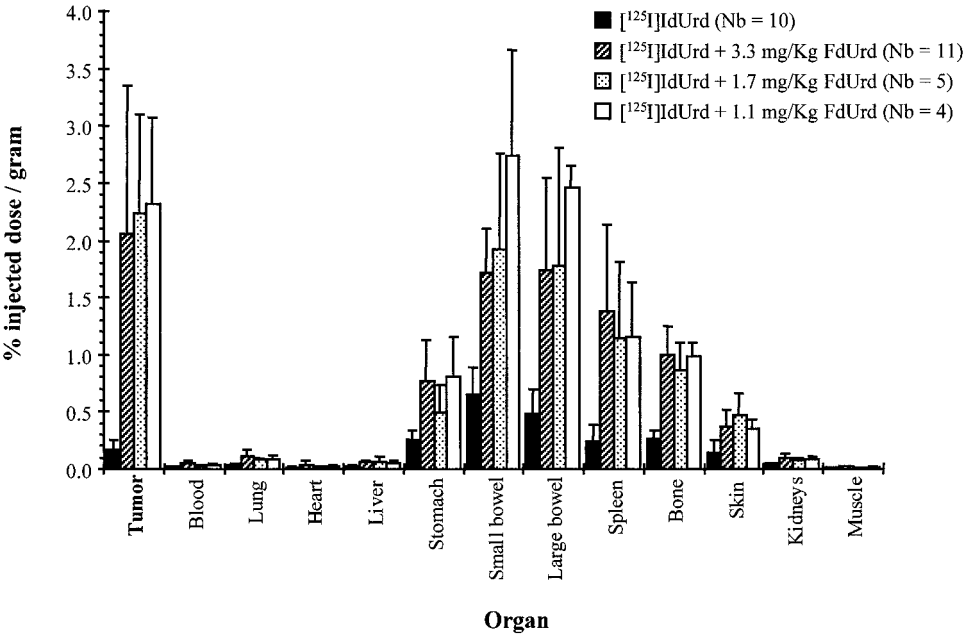

Fig. 4. Biodistribution of [^{125}I]IdUrd in nude mice xenografted with LN229. Each experiment included a control group of mice injected i.v. with 185 KBq [^{125}I]IdUrd. FdUrd was administered by two i.v. injections 60 and 30 min before i.v. injection of 185 KBq [^{125}I]IdUrd. Mice were dissected 24 h after [^{125}I]IdUrd injection. Data are presented as the percentage of injected dose per gram of tissue ±1 SD. *Nb*, number of mice.

Table 3 *Incorporation rate of [^{125}I]IdUrd in DNA of tumor and intestine*

	%A[a] in tumor DNA	%A in intestine DNA	*P*
[^{125}I]IdUrd	72.1 ± 8.1	79.5 ± 7.3	0.28
[^{125}I]IdUrd + 3.3 mg/kg FdUrd	77.9 ± 11.3	77.7 ± 9.4	0.98
P	0.50	0.81	

[a] %A, percentage of DNA associated activity ± 1 SD, determined in tissues of animals dissected 24 h after administration of [^{125}I]IdUrd (total activity = 100%).

a high spontaneous apoptotic activity in intestine. The lack of toxicity was confirmed in mice observed for 2 months after i.v. injections of 10 mg/kg FdUrd in which no side effect could be detected.

In agreement with previous observations (5), high amounts of free iodine-125 precluded any correlation between cell division and tissue radioactivity when [^{125}I]IdUrd was injected only 6 h before biodistribution measurements (results not shown). Therefore, all subsequent biodistribution studies were carried out 24 h after [^{125}I]IdUrd injection to profit from the decrease of unspecific background radioactivity by renal elimination of degradation products, whereas specific uptake of [^{125}I]IdUrd should remain at the level observed 1 h after its bolus i.v. injection, according to a previous report (17).

Using such a schedule, we show that FdUrd preinjection greatly increases uptake of [^{125}I]IdUrd in xenografted glioblastoma tumors, compared with [^{125}I]IdUrd injection alone. This observation is in agreement with a study with xenografted HT29 colon carcinoma tumors demonstrating an increase in DNA-incorporation of unlabeled IdUrd above that obtained with IdUrd alone (14). Furthermore, we observed more favorable tumor:normal tissue ratios after FdUrd preinjection, attributable to slightly lower uptake increases of [^{125}I]IdUrd in normal dividing tissues, whereas background activity in nondividing tissues, such as brain, liver, lung, and kidneys, remained unchanged. The higher uptake increase of [^{125}I]IdUrd in tumors as compared with normal proliferating tissues, also observed with another dThd inhibitor, methotrexate (17, 18), is encouraging but difficult to understand. DNA extraction analysis showed that the difference in uptake increase between tumor and intestine could not be attributable to variations in background radioactivity, because similar high percentages of radioactivity were incorporated into DNA, both in controls and animals preinjected with FdUrd. Similarly, a difference in *p53* status of tumor and intestine cannot be responsible for this effect, because both wild-type and mutant *p53* glioblastoma lines showed comparable incorporation increases after FdUrd. As a last hypothesis, normal tissues might present some resistance to inhibition of TS by FdUrd, which could be attributable to a decreased anabolism of fluoropyrimidines into FdUMP, a lowered affinity of target enzyme for FdUMP, or expanded intracellular TS levels (33). This latter hypothesis is supported by a previous observation, that radioactivity was maintained and even increased in tumor 4–24 h after [^{14}C]FdUrd

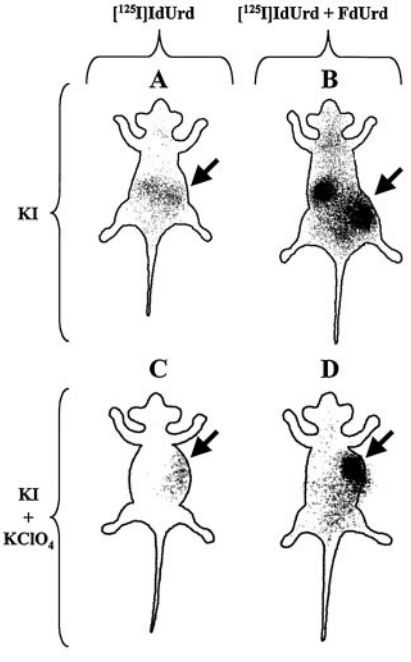

Fig. 5. Whole body scintigraphy 24 h after i.v. injection of 3.7 MBq [^{125}I]IdUrd in nude mice xenografted with LN229 tumor of comparable tumor size. Before scintigraphy (24 h), mice A and B received 0.2 grams/liter KI in drinking water, whereas mice C and D received 2 grams/liter $KClO_4$ in addition to KI in drinking water. Mice A and C were injected with [^{125}I]IdUrd alone, whereas mice B and D were preinjected i.v. with 3.3 mg/kg FdUrd before injection of [^{125}I]IdUrd. Scintigraphy of anesthetized mice was conducted with a LEHR-parallel collimator on GCA 9300 UI Toshiba camera. *Arrow*, tumor localization.

exposure, whereas it was decreased in other dividing tissues, such as bone marrow and small intestine (26).

The observation of a significantly improved tumor uptake of radio-IdUrd after FdUrd prompted us to conduct comparative scintigraphy studies on mice treated or not with FdUrd. The timing and accuracy of tumor detection could be clearly improved in FdUrd-treated animals compared with control mice. Still, a limiting factor consisted initially of an elevated residual background radioactivity, most pronounced in stomachs of both control- and FdUrd-treated mice. Background activity attributable to degradation products could mask tumor detection in abdominal tissues or contribute to total activity even in brain tumors (1, 6). Dissection and gamma counting showed that radioactivity was concentrated in the stomach content rather than in the stomach wall. Because stomach secretion and intestine reabsorption of free radio-iodine have been shown to constitute an iodine reservoir that competes with and can delay renal elimination, we conducted biodistribution studies to evaluate the potential of $KClO_4$ to block stomach secretion of free radio-iodine and accelerate its renal elimination. Stomach secretion as well as whole body retention of free radio-iodine were markedly reduced 4 h after injection in mice preadministered $KClO_4$ in addition to KI. Thus, in the subsequent scintigraphy studies, the additional administration of $KClO_4$ allowed demonstration of the full potential of FdUrd for increasing the specific tumor uptake of [^{125}I]IdUrd, by improving the contrast of tumor imaging with less background.

Scintigraphy using [^{124}I]IdUrd has recently been applied to patients with the aim to image proliferation of malignant brain lesions. The main limitations have been shown to be low specific uptake of radio-IdUrd together with high background in tumor, especially in low-grade meningioma where background activity as late as 24 h after injection still contributed to 85% of overall tumor radioactivity (6). Our results strongly suggest that a combination with FdUrd together with $KClO_4$ administration could significantly improve such tumor detection with radio-IdUrd or other radio-dThd analogues as tracers in positron or single photon emission tomography imaging.

As a limitation, however, IdUrd and probably also FdUrd, do not cross the intact blood-brain barrier. Despite this limitation, different brain tumors have been successfully imaged as well with [^{124}I]IdUrd as with [^{18}F]FdUrd and influx rates calculated (2, 6). Indeed, the blood-brain barrier is frequently disrupted in these tumors. Moreover, the combined use of FdUrd and radio-IdUrd for diagnostic imaging is not limited to brain tumors, because increase in DNA-incorporation of IdUrd has already been observed *in vitro* for other types of tumors (28).

Overall, we have shown *in vitro* and *in vivo* that FdUrd is able to significantly enhance incorporation of radio-IdUrd into glioblastoma cells and tumors. As a result, the tumor localization in xenografted mice by scintigraphy is greatly improved, suggesting that this approach might be clinically useful.

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REFERENCES

1. Eary, J. F., Mankoff, D. A., Spence, A. M., Berger, M. S., Olshen, A., Link, J. M., O'Sullivan, F., and Krohn, K. A. 2-[C-11]thymidine imaging of malignant brain tumors. Cancer Res., *59:* 615–621, 1999.
2. Kameyama, M., Ishiwata, K., Tsurumi, Y., Itoh, J., Sato, K., Katakura, R., Yoshimoto, T., Hatazawa, J., Ito, M., and Ido, T. Clinical application of ^{18}F-FUdR in glioma patients-PET study of nucleic acid metabolism. J. Neuro-Oncol., *23:* 53–61, 1995.
3. Shields, A. F., Grierson, J. R., Dohmen, B. M., Machulla, H-J., Stayanoff, J. C., Lawhorn-Crews, J. M., Obradovich, J. E., Muzik, O., and Mangner, T. J. Imaging proliferation *in vivo* with [F-18]FLT and positron emission tomography. Nat. Med., *4:* 1334–1336, 1998.
4. Bergström, M., Lu, L., Fasth, K-J., Wu, F., Bergström-Pettermann, E., Tolmachev, V., Hedberg, E., Cheng, A., and Längström, B. *In vitro* and animal validation of bromine-76-bromodeoxyuridine as a proliferation marker. J. Nucl. Med., *39:* 1273–1279, 1998.
5. Ryser, J. E., Blauenstein, P., Remy, N., Weinreich, R., Hasler, P. H., Novak-Hofer, I., and Schubiger, P. A. [^{76}Br]Bromodeoxyuridine, a potential tracer for the measurement of cell proliferation by positron emission tomography, *in vitro* and *in vivo* studies in mice. Nucl. Med. Biol., *26:* 673–679, 1999.
6. Blasberg, R. G., Roelcke, U., Weinreich, R., Beattie, B., von Ammon, K., Yonekawa, Y., Landolt, H., Guenther, I., Crompton, N. E. A., Vontobel, P., Missimer, J., Maguire, R. P., Koziorowski, J., Knust, E. J., Finn, R. D., and Leenders, K. L. Imaging brain tumor proliferative activity with [^{124}I]iododeoxyuridine. Cancer Res., *60:* 624–635, 2000.
7. Foulon, C. F., Zhang, Y. Z., Adelstein, S. J., and Kassis, A. I. Instantaneous preparation of radiolabeled 5-iodo-2′-deoxyuridine. Appl. Radiat. Isot., *46:* 1039–1046, 1995.
8. Commerford, S. L. Biological stability of IUdR labeled with ^{125}I after incorporation into the DNA of the mouse. Nature (Lond.), *206:* 949–950, 1965.
9. Klecker, R. W. J., Jenkins, J. F., Kinsella, T. J., Fine, R. L., Strong, J. M., and Collins, J. M. Clinical pharmacology of 5-iodo-2′-deoxyuridine and 5-iodouracil and endogenous pyrimidine modulation. Clin. Pharmacol. Ther., *38:* 45–51, 1985.
10. Xiao, W-H., Dupertuis, Y. M., Mermillod, B., Sun, L-Q., De Tribolet, N., and Buchegger, F. Unlabelled iododeoxyuridine increases the cytotoxicity and incorporation of ^{125}I-iododeoxyuridine in 2 human glioblastoma cell lines. Nucl. Med. Commun., *21:* 947–953, 2000.
11. Armstrong, R. D., and Diasio, R. B. Metabolism and biological activity of 5′-deoxy-5-fluorouridine, a novel fluoropyrimidine. Cancer Res., *40:* 3333–3338, 1980.
12. Brown, J. M., Goffinet, D. R., Cleaver, J. E., and Kallman, R. F. Preferential radiosensitization of mouse sarcoma relative to normal skin by chronic intra-arterial infusion of halogenated pyrimidine analogs. J. Natl. Cancer Inst. (Bethesda), *47:* 75–89, 1971.
13. Benson, A. B. I., Trump, D. L., Cummings, K. B., and Fischer, P. H. Modulation of 5-iodo-2′-deoxyuridine metabolism and cytotoxicity in human bladder cancer cells by fluoropyrimidines. Biochem. Pharmacol., *34:* 3925–3931, 1985.
14. Lawrence, T. S., Davis, M. A., McKeever, P. E., Maybaum, J., Stetson, P. L., Normolle, D. P., and Ensminger, W. D. Fluorodeoxyuridine-mediated modulation of iododeoxyuridine incorporation and radiosensitization in human colon cancer cells *in vitro* and *in vivo*. Cancer Res., *51:* 3900–3905, 1991.
15. Mancini, W. R., Stetson, P. L., Lawrence, T. S., Wagner, J. G., Greenberg, H. S., and Ensminger, W. D. Variability of 5-bromo-2′-deoxyuridine incorporation into DNA of human glioma cell lines and modulation with fluoropyrimidines. Cancer Res., *51:* 870–874, 1991.
16. Kassis, A. I., Guptill, W. E., Taube, R. A., and Adelstein, S. J. Radiotoxicity of 5-[^{125}I]iodo-2′-deoxyuridine in mammalian cells following treatment with 5-fluoro-2′-deoxyuridine. J. Nucl. Biol. Med., *35:* 167–173, 1991.
17. Mester, J., De Goeij, K., and Sluyser, M. Modulation of [5-^{125}I]iododeoxyuridine incorporation into tumour and normal tissue DNA by methotrexate and thymidylate synthase inhibitors. Eur. J. Cancer, *32A:* 1603–1608, 1996.
18. Kassis, A. I., Dahman, B. A., and Adelstein, S. J. *In vivo* therapy of neoplastic meningitis with methotrexate and 5-[^{125}I]iodo-2-deoxyuridine. Acta Oncol., *39:* 731–737, 2000.
19. Van Meir, E. G., Kikuchi, T., Tada, M., Li, H., Diserens, A-C., Wojcik, B. E., Su Huang, H-J., Friedmann, T., de Tribolet, N., and Cavenee, W. K. Analysis of the *p53* gene and its expression in human glioblastoma cells. Cancer Res., *54:* 649–652, 1994.
20. Ali, I. U., Schweitzer, J. B., Ikejiri, B., Saxena, A., Robertson, J. T., and Oldfield, E. H. Heterogeneity of subcellular localization of *p53* protein in human glioblastomas. Cancer Res., *54:* 1–5, 1994.
21. Tada, M., Matsumoto, R., Iggo, R. D., Onimaru, R., Shirato, H., Sawamura, Y., and Shinohe, Y. Selective sensitivity to radiation of cerebral glioblastomas harboring *p53* mutations. Cancer Res., *58:* 1793–1797, 1998.
22. Van Meir, E. G., Roemer, K., Diserens, A-C., Kikuchi, T., Rempel, S. A., Haas, M., Su Huang, H-J., Friedmann, T., De Tribolet, N., and Cavenee, W. K. Single cell monitoring of growth arrest and morphological changes induced by transfer of wild-type *p53* alleles to glioblastoma cells. Proc. Natl. Acad. Sci. USA, *92:* 1008–1012, 1995.
23. Kassis, A. I., Van den Abbeele, A. D., Wen, P. Y. C., Baranowska-Kortylewicz, J., Aaronson, R. A., DeSisto, W. C., Lampson, L. A., Black, P. M., and Adelstein, S. J. Specific uptake of the Auger electron-emitting thymidine analogue 5-[^{123}I/^{125}I]iodo-2′-deoxyuridine in rat brain tumors: Diagnostic and therapeutic implications in humans. Cancer Res., *50:* 5199–5203, 1990.
24. Kassis, A. I., Tumeh, S. S., Wen, P. Y. C., Baranowska-Kortylewicz, J., Van den Abbeele, A. D., Zimmerman, R. E., Carvalho, P. A., Garada, B. M., DeSisto, W. C., Bailey, N. O., Castronovo, F. P. J., Mariani, G., Black, P. M., and Adelstein, S. J. Intratumoral administration of 5-[^{123}I]iodo-2′-deoxyuridine in a patient with a brain tumor. J. Nucl. Med., *37* (Suppl. 4)*:* 19S–22S, 1996.
25. Hogan, B., Beddington, R., Costantini, F., and Lacy, E. (eds.). Manipulating the Mouse Embryo, pp. 296–298. Cold Spring Harbor Laboratory, NY: Cold Spring Harbor Laboratory, 1994.
26. Harbers, E., Chaudhuri, N. K., and Heidelberger, C. Studies on fluorinated pyrimidines. J. Biol. Chem., *234:* 1255–1262, 1959.

27. Greene, R. F., and Collins, J. M. Effects of leucovorin on idoxuridine cytotoxicity and DNA incorporation. Cancer Res., *50:* 6652–6656, 1990.
28. Miller, E. M., and Kinsella, T. J. Radiosensitization by fluorodeoxyuridine: effects of thymidylate synthase inhibition and cell synchronization. Cancer Res., *52:* 1687–1694, 1992.
29. Canman, C. E., Lawrence, T. S., Shewach, D. S., Tang, H-Y., and Maybaum, J. Resistance to fluorodeoxyuridine-induced DNA damage and cytotoxicity correlates with an elevation of deoxyuridine triphosphatase activity and failure to accumulate deoxyuridine triphosphate. Cancer Res., *53:* 5219–5224, 1993.
30. Djordjevic, B., and Szybalski, W. Genetics of human cell lines. III. Incorporation of 5-bromo and 5-iododeoxyuridine into the deoxyuribonucleic acid of human cells and its effect on radiation sensitivity. J. Exp. Med., *112:* 509–531, 1960.
31. Port, J. L., Ng, B., Ellis, J. L., Nawata, S., Lenert, J. T., and Burt, M. E. Isolated lung perfusion with FUdR in the rat: pharmacokinetics and survival. Ann. Thorac. Surg., *62:* 848–852, 1996.
32. Morgan, R. J. J., Newman, E. M., Doroshow, J. H., McGonigle, K., Margolin, K., Raschko, J., Chow, W., Somlo, G., Leong, L., Tetef, M., Shibata, S., Hamasaki, V., Caroll, M., Vasilev, S., Akman, S., Coluzzi, P., Wagman, L., Longmate, J., Paz, B., Yen, Y., and Klevecz, R. Phase I trial of intraperitoneal iododeoxyuridine with and without intravenous high-dose folinic acid in the treatment of advanced malignancies primarily confined to the peritoneal cavity: flow cytometric and pharmacokinetic analysis. Cancer Res., *58:* 2793–2800, 1998.
33. Grem, J. L. Fluorinated pyrimidine. *In:* B. A. Chabner and J. M. Collins (eds.), Cancer Chemotherapy: Principles and Practice, pp. 180–224. Philadelphia: J. B. Lippincott Co., 1990.