A Novel, Orally Administered Nucleoside Analogue, OGT 719, Inhibits the Liver Invasive Growth of a Human Colorectal Tumor, C170HM2

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ABSTRACT

OGT 719 is a novel p.o. bioavailable nucleoside analogue in which galactose is incorporated onto the fluoropyrimidine moiety of the cytotoxic agent 5-fluorouracil (5-FU). OGT 719 has been designed to reduce the systemic toxicity normally associated with 5-FU while retaining activity against disease localized in the liver, in which it may be preferentially localized through the asialoglycoprotein receptor (ASGP-R). We report studies confirming the activity of OGT 719 in inhibiting growth of metastatic human colorectal tumors in the liver of nude mice. The human colorectal cancer cell line C170HM2 readily forms liver metastases in vivo. Oral administration of 1500 mg/kg/day OGT 719 inhibited liver tumor burden by 95% compared with vehicle control, without any observable signs of toxicity. When the tumor burden was increased and the same OGT 719 treatment was compared with a standard clinical dose regimen of 25 mg/kg/day 5-FU/leucovorin given i.v., both treatments were equally efficacious, although 5-FU/leucovorin treatment started 7 days earlier. In contrast to 5-FU, OGT 719 is p.o. bioavailable and has a plasma half-life between 1.5 and 3 h. Several colorectal cancer cell lines express the asialoglycoprotein receptor, although no significant levels can be detected in C170HM2 cells, consistent with the observation that OGT 719 is approximately 3 log orders of magnitude less potent in vitro than 5-FU.

INTRODUCTION

Colorectal cancer is the second leading cause of cancer-related death (1). The liver is a dominant site for metastatic spread from primary colorectal tumors, with 60% of patients having hepatic spread (2, 3). This is usually the cause of fatality in such patients. The standard treatment regimens for colorectal cancer are various combinations of systemic 5-FU (4) and the biomedical anti-folate Lv (reviewed in Refs. 4–6). Adjuvant therapy with high-dose 5-FU and Lv has also been explored successfully (reviewed in Ref. 7). 5-FU cytotoxicity depends on conversion to the nucleotides 5-fluoro-UTP and 5-fluoro-dUMP, via incorporation into RNA and binding to TS (4), respectively, in the presence of reduced folate cofactor. The latter effect results in the inhibition of thymidine nucleotide synthesis (8). The inhibition of DNA synthesis via inhibition of TS and its incorporation into RNA probably both contribute to antitumor activity (reviewed in Ref. 9). Furthermore, 5-FU-induced cell death of intestinal epithelial cells appears to involve changes in RNA metabolism (8).

Recent efforts to improve the toxicity profile of 5-FU have been directed toward a prodrug formulation of 5-FU, i.e., capecitabine (10) and tegafur. A combination of uracil, tegafur, and Lv has an improved toxicity profile compared with 5-FU and Lv alone (11). In patients with colorectal cancer, tumor concentration of 5-FU appears to be higher after venous injection with tegafur compared with an injection with 5-FU alone. This is quite surprising, because the plasma levels of 5-FU in the patients treated with 5-FU were ~27-fold higher than in patients that received tegafur (12). A strategy for a tumor-specific prodrug uses an enzyme/prodrug gene therapy approach for the tumor-specific conversion of 5-fluorocytosine (4) to 5-FU in metastatic colorectal cancer (13).

Alternatively, one could target 5-FU to the tumor or its host organ via unique cell surface receptors. The liver-specific, carbohydrate-binding ASGP-R is known to be present on the surface of hepatocytes (14, 15). The ASGP-R has a major role in the clearance of desialylated proteins, such as asialoorosomucoid from the general circulation, resulting in their accumulation within the liver lysosomes. The recognition sites for the ASGP-R include terminal galactose residues and N-acetylgalactosamine (16). Although its mRNA has been detected in a variety of tissues (17), functional ASGP-R protein is found almost exclusively on hepatocytes (18). Extrahepatic ASGP-R has been described in testicular sertoli cells (19, 20). A galactose-specific receptor on rat liver macrophages appears not to be structurally related to the hepatocyte receptor (21, 22). Among tumor cells, functional ASGP-R has been detected in the human hepatoma Huh 7 and HepG2 cell lines (23, 24), Jurkat T-cell line (25), and the human colonic adenocarcinoma Caco-2 and HT-29 cell lines (26). Therefore, the possibility exists that malignancies arising within the gastrointestinal epithelium may have increased ASGP-R expression. We have reasoned that a cytotoxic agent (such as 5-FU) modified chemically to carry a ligand for the ASGP-R may show greater cytotoxicity against cancer cells carrying the ASGP-R and correspondingly reduced cytotoxicity against host cells lacking this receptor.

The aim of this study was to evaluate the effect of OGT 719 on the liver-invasive growth of metastatic colorectal tumor cells. OGT 719 is a nucleoside analogue composed of a 5-FU pyrimidine base to which a galactose carbohydrate moiety is covalently attached (Fig. 1). To investigate this compound, the C170HM2 human colorectal tumor cell line was chosen because this has been selected to invade and grow within the liver (27). This cell line has been used previously for the in vivo assessment of experimental drugs (28, 29). Our findings demonstrate that p.o.-administered OGT 719 has a major inhibitory effect on liver-established C170HM2 tumors in vivo, without any observable toxicity.

MATERIALS AND METHODS

Materials. OGT 719 [Oxford GlycoSciences (UK) Ltd.] and 5-FU (Sigma, Poole, United Kingdom) were made up in distilled water. 5-FU and dUMP (Sigma) were prepared as 20 and 10 mM solutions, respectively, in 0.15 M sodium bicarbonate. A folate-based TS inhibitor used as a control reagent was

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2 The abbreviations used are: Lv, leucovorin; 5-FU, 5-fluorouracil; ASGP-R, asialoglycoprotein receptor; dUrd, deoxyuridine; IC 50, 50% inhibitory concentration; TS, thymidylate synthase.
made up at 10 mM in 0.15 M sodium bicarbonate (30). All stock solutions were diluted as required in distilled water. 5-[^3]HdUMP and 5-[^3]HdUrd were obtained from Moravek Biochemicals, Inc. (Brea, CA).

Cell Lines. The human colon carcinoma cell line C170HM2 was derived originally from a primary tumor (27). C170HM2 cells were cultured in RPMI 1640 (Life Technologies, Inc., Paisley, United Kingdom) supplemented with 10% FCS (Sigma) at 37°C in a humidified incubator in a 5% CO2, 95% air atmosphere.

Growth Inhibition Assays. C170HM2 cells were seeded in 96-well plates (Flow Labs, Irvine, Scotland) at a cell concentration of 2 x 103/well, incubated to adhere overnight, and then treated with concentrations (each in quadruplicate) ranging from 1 μM to 50 μM of 5-FU or OGT 719. After 120 h, cell growth was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (31), and IC50 values were determined.

Preparation of Cell Membrane Fractions. Log phase growing and confluent cells were harvested with a cell lifter in ice-cold PBS and resuspended in 10 mM Tris (pH 7.2) containing a protease inhibitor mixture (Boehringer Mannheim). The cell suspensions were then sonicated in two 10-s intervals on ice. After a 10-min spin at 1000 x g, membrane fractions were collected from the supernatant via ultracentrifugation at 100,000 x g for 60 min.

Immunoblot of the ASGP-R H1 Subunit. Thirty μg of membrane proteins denatured in 1 x Laemmli buffer were separated on Tris-glycine SDS-PAGE with a 4–20% acrylamide gradient (Novex). Proteins were blotted onto a polyvinylidene difluoride membrane (Novex) and immunoblotted with a polyclonal antibody raised against the ASGP-R H1 subunit (kindly provided by Dr. Richard Stockert) in 3% dry milk. Secondary detection with a monoclonal anti-rabbit immunoglobulin-peroxidase conjugate (Sigma) was performed with the Chemiluminescence Reagent Plus system (NEN Life Science).

Inhibition of Isolated TS. TS activity was measured by the 3H-release assay (32). This involves incubating TS (partially purified from mouse L1210: C170 cells; Ref. 33) at 37°C for 1 h with a range of concentrations of OGT 719, 200 μM 5,10-methylene tetrahydrofolate, and 50 μM [3H]dUMP (specific activity, 50 μCi/mmole). The product, tritiated H2O, is collected after passage of the supernatant via ultracentrifugation at 100,000 x g for 60 min.

RESULTS

Effect of OGT 719 on C170HM2 Liver Tumor Burden. Inoculation of 1 x 106 C170HM2 cells led after 40 days to mice in the untreated (water) control group developing a mean liver tumor burden of 0.426 g/mouse, which was similar to that seen in previous studies with this model (28; Fig. 2). The tumor incidence in this control group was 13 of 18 animals, and in the OGT 719-treated group, the tumor incidence was 1 of 18 animals. This substantial reduction as a result of OGT 719 treatment is particularly notable because tumors were allowed to establish for 10 days before treatment. The inhibitory effect of OGT 719 was also reflected in a substantial reduction in the mean C170HM2 liver tumor burden after treatment with OGT 719, as shown by a Mann Whitney U nonparametric test (0.426 g reduced to 0.024 g, P = 0.0006), shown in Fig. 2. There was also a significant reduction (93%) in the mean liver tumor cross-sectional area (79.39 mm2 down to 5.53 mm2; P = 0.0002, Mann Whitney). Despite the intensive dosing regimen, there was no detrimental effect of OGT 719 on the condition of the mice, as recorded by observation and body weight gain (data not shown).

A Comparative Analysis of OGT 719 and 5-FU/Lv on C170HM2 Liver Tumor Burden. OGT 719 was compared with a recognized chemotherapeutic regimen used to treat colorectal liver metastases. In this experiment, the tumor burden was increased by raising the cell inoculation to 1.5 x 106 cells. Following a 41-day growth period in the mice, the mean control tumor burden was 1.81 g. Even with this elevated tumor burden, there was a significant 70% reduction in mean liver tumor burden in the OGT 719-treated group (Fig. 3; 1.81 g reduced to 0.54 g; P = 0.0225, Mann Whitney). 5-FU/Lv treatment given i.v. decreased the mean tumor burden 60.7% (1.81 reduced to 0.71 g; P = 0.0465, Mann Whitney), although treatment started 7 days earlier than the OGT 719 therapy (Fig. 3).

In Vivo Studies. C170HM2 cell suspensions were injected into the peritoneal cavity of male MFI nude mice (bred within the Cancer Studies Unit at the University of Nottingham) in 1 ml of 0.9% sodium chloride (pH 7.3). Mice were electronically tagged for identification purposes (RS Biotech; DL2000 Datalogger) and randomly allocated to experimental groups. In the first experiment, 1 x 106 C170HM2 cells were inoculated into the peritoneal cavity of each of 36 mice. Ten days after tumor inoculation, 18 mice received water, and 18 mice were treated with OGT 719 at a dose of 1500 mg/kg/twice a day and sacrificed on day 40, by terminal anesthesia. In the second experiment, the tumor inoculum was increased to 1.5 x 106 cells. Three experimental groups of 15 mice received either vehicle control (p.o. twice daily), 1500 mg/kg/day OGT 719 (p.o. twice daily), or 5-FU/Lv, 25 mg/kg each, i.v. on days 3, 5, and 7, and 10 (cycle repeated from day 28). p.o.-dosed mice were treated twice daily as above, from day 10 to sacrifice on day 42. The mice were terminally anesthetized, and heparinized samples were taken by cardiac puncture. Plasma was separated and stored at -80°C. The liver was exposed, excised, and analyzed as described (27).
The 5-FU/Lv treated mice (324.52 mm$^2$ reduced to 135.47 mm$^2$; OGT 719 ranging from 0 to 100 mM dissolved in water. Each cell line was treated with tested for their sensitivity to OGT 719 in vitro (as assessed by analysis of variance, data not shown).

C170HM2 cells used in the animal experiments were C170HM2 cells, with an IC$_{50}$ of 2.7 $\mu$m in the liver. Results are shown in Table 1. 5-FU is moderately potent in the range of 20–30 $\mu$m (Table 1).

OGT 719 to be $\sim$20% in a rat model. To assess differences in murine gastrointestinal metabolism of OGT 719, an abbreviated bioavailability study was undertaken. OGT 719 plasma levels in mice after oral administration of 1500 mg/kg/day twice daily for 7 days were in the gastrointestinal metabolism of OGT 719, an abbreviated bioavailability study was undertaken. OGT 719 plasma levels in mice after oral administration of 1500 mg/kg/day twice daily for 7 days were in the range of 20–30 $\mu$m within the first 6 h after the last gavage (data not shown).

In Vitro Comparison of the Antiproliferative Effects of OGT 719 and 5-FU. C170HM2 cells used in the animal experiments were tested for their sensitivity to OGT 719 in vitro. Several colorectal cancer cells described in the literature express the ASGP-R (26), suggesting that growth of these cells may be directly inhibited by OGT 719, rather than through active metabolites generated in a mouse liver. Results are shown in Table 1. 5-FU is moderately potent in the C170HM2 cells, with an IC$_{50}$ of 2.7 ± 0.5 $\mu$m. The IC$_{50}$ for OGT 719 in these cells was much higher, $\sim$20–40 $\mu$m (Table 1).

ASGP-R Levels in C170HM2 Cell Membranes. One explanation for the lack of potency of OGT 719 in vitro is an absence of ASGP-R in these cells. Therefore, we determined the levels of the ASGP-R H1 subunit in these cells. Although the ASGP-R preferentially forms H1:H2 heterotetramers in vitro (38), H1 is the dominant subunit detected in hepatoma cells and an adenocarcinoma cell line from the human colon (26). In the latter cells, receptor levels doubled in confluent cell cultures. Furthermore, H1-overexpressing cells lacking H2 can bind ASGP-R ligands (39).

An examination of H1 levels present in crude membranes isolated from C170HM2 cells revealed no significant H1. Although a weak immunoreactive band is present at $M_r \sim$46,000 similar in size to the human ASGP-R H1 species (40) found in human Huh7 hepatoma cells, no increase was observed in cells cultured to confluence for 4 days (Fig. 4). A modest increase of H1 in confluent versus log phase growing hepatoma cells was observed both under normal and low FCS conditions.

Inhibition of Partially Purified TS. It is believed that two 5-FU metabolites (5-fluoro-UTP and 5-fluoro-dUMP) contribute to the cytotoxic effect of 5-FU via incorporation into RNA and inhibition of TS, respectively, to a varying extent in different cell types (9). It is known that in some colorectal cells, TS inhibition significantly accounts for 5-FU cytotoxic activity, leading to inhibition of DNA synthesis. To determine if OGT 719 is a direct TS inhibitor itself, activity of partially purified TS was measured in the absence and presence of OGT 719 or 5-FU. OGT 719 was inactive against isolated TS at a concentration of 3.4 mM. 5-FU, the produg form ofFdUMP, was also inactive at mM concentrations, whereas 5-FdUMP gave an IC$_{50}$ of $\sim$0.1 $\mu$m (data not shown).

Effect of 5-FU and OGT 719 on TS in Tumor Cells. 5-FU requires metabolic activation inside cells to become a cytotoxic. To assess if TS inhibition contributes to 5-FU- or OGT 719-mediated cytotoxicity, a cell-based TS assay was performed in the C170HM2 cells. At concentrations approximately two times IC$_{50}$, $^3$H release from $[^3]$H)dUrd activity was inhibited with OGT 719 only after 4 h (Table 2). These data suggest that OGT 719 or a metabolite thereof, but not 5-FU, inhibits TS in C170HM2 cells.

Fig. 3. Comparison of OGT 719 and 5-FU/Lv on the liver burden of human colorectal tumor in vivo. Mice were inoculated with of $1.5 \times 10^6$ C170HM2 cells, as described in “Materials and Methods.” The average tumor burden for each experimental group is shown; bars, SE. Control (C), vehicle control. OGT 719, OGT 719 at 1500 mg/kg/day, p.o., twice daily. 5-FU/Lv, 25 mg/kg 5-FU/Lv, each i.v., on days 3, 5, 7, and 10, with the cycle repeated every 28 days. The percentage of mean inhibition of mean tumor burden compared with vehicle control was 70% for OGT 719 ($P = 0.0225$) and was 61% for 5-FU/Lv ($P = 0.0465$).

Table 1 Cytotoxicity of 5-FU and OGT 719 in the C170HM2 cell line

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Drug incubation time</th>
<th>IC$_{50}$</th>
</tr>
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<tbody>
<tr>
<td>C170HM2</td>
<td>5-FU</td>
<td>2.7 ± 0.5 $\mu$m</td>
</tr>
<tr>
<td></td>
<td>120 h</td>
<td>19 $\mu$m</td>
</tr>
<tr>
<td></td>
<td>OGT 719</td>
<td>39 $\mu$m</td>
</tr>
<tr>
<td></td>
<td>120 h</td>
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</table>

There was no statistically significant difference between the two treatment groups ($P = 0.561$, Mann Whitney).

As in the first experiment, there was a significant decrease in mean liver tumor cross-sectional areas in both the OGT 719-treated mice (324.52 mm$^2$ reduced to 98.15 mm$^2$; $P = 0.0101$, Mann Whitney) and the 5-FU/Lv treated mice (324.52 mm$^2$ reduced to 135.47 mm$^2$; $P = 0.031$, Mann Whitney) when compared with the vehicle controls. There was no difference in the liver tumor cross-sectional areas of the two treatment groups ($P = 0.619$, Mann Whitney). Neither treatment had a significant effect on animal weight over the duration of the study (as assessed by analysis of variance, data not shown).

Although 5-FU is p.o. bioavailable, its pharmacokinetics after oral administration vary greatly and preclude its use in the clinic via this route (36, 37). We have determined the oral bioavailability of OGT 719 to be $\sim$20% in a rat model. To assess differences in murine gastrointestinal metabolism of OGT 719, an abbreviated bioavailability study was undertaken. OGT 719 plasma levels in mice after oral administration of 1500 mg/kg/day twice daily for 7 days were in the range of 20–30 $\mu$m within the first 6 h after the last gavage (data not shown).

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OGT 719 INHIBITS LIVER GROWTH OF COLORECTAL TUMOR

Table 2  OGT 719 but not 5-FU inhibits TS activity in C170HM2 colorectal cancer cells

<table>
<thead>
<tr>
<th>Drug</th>
<th>TS activity rate (pmol/min/10^6 cells)</th>
<th>Rate as % of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.41 ± 0.024</td>
<td></td>
</tr>
<tr>
<td>5-FU, 6 μM (2 × IC_{50})</td>
<td>0.55 ± 0.050</td>
<td></td>
</tr>
<tr>
<td>OGT 719, 80 μM (2 × IC_{50})</td>
<td>0.60 ± 0.050</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.25 ± 0.022</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td>0.31 ± 0.022</td>
<td>56</td>
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</table>

DISCUSSION

Pyrimidine antimetabolites remain the focus of new treatments in metastatic malignancies of the colon. Recent examples include difluorodeoxycytidine (gemcitabine) and the 5-FU prodrug tegafur (12). Folinic acid modulation of 5-FU increases the response rate without affecting survival (41). All three therapeutic approaches are still associated with dose-limiting toxicities, including myelosuppression, nausea, alopecia, and fever in the case of gemcitabine (42), mucositis and diarrhea in 5-FU/Lv and oral capecitabine or tegafur/Lv treatments (10, 11). There remains a genuine need for tumor-specific, p.o.-available prodrugs that can maintain the efficacy of the parent drug while substantially reducing its systemic toxicity.

In an attempt to achieve this, we have designed and selected OGT 719, a novel nucleoside (5-FU) analogue containing a galactose moiety chosen for its affinity to the ASGP-R. We present here our evaluation of OGT 719 in modulating tumor burden in a human colorectal (C170HM2) model, which localizes to the mouse liver. p.o.-administered OGT 719 has a high inhibitory effect on the liver growth of this tumor and is equal in its efficacy to an i.v. 5-FU/Lv regimen similar to that commonly used in the clinic, although tumors in the OGT 719-treated mice had been allowed to establish and grow 1 week longer before treatment started. The lack of tumors in the livers of the majority of mice in the OGT 719-treated group indicates that tumor regression and/or resolution may have occurred. Consistent with this, white nodules, too small to dissect, were observed repeatedly in the liver of OGT 719-treated mice and gave the appearance of being necrotic (data not shown).

We observed a notable difference in the potency of OGT 719 on C170HM2 cells in vitro and in vivo. This suggests that the cytotoxic action of OGT 719 may be enhanced by uptake and metabolism in mouse hepatocytes, with release of active metabolites that exert a cytotoxic effect on adjacent tumors, i.e., a localized bystander effect. In support of this, we have observed that C170HM2 cells do not express significant levels of ASGP-R. A benefit of a bystander mechanism may be negligible cytotoxicity of quiescent hepatocytes in the host liver.

In these studies, only OGT 719 inhibited TS activity in tissue culture cells at ~2 × IC_{50} for 4 h. Also, because high concentrations of OGT 719 are used, we cannot eliminate that the TS inhibitory effects could be mediated by alternative mechanisms, such as transporters. It remains to be determined whether the TS inhibitory component of OGT 719 is the dominant pathway to antitumor activity in colorectal cancers in vivo. The complex manner by which 5-FU prodrugs become activated has been exemplified by the metabolism of FdUrd in vitro and in vivo. In cultured cancer cells, FdUrd is mainly metabolized via one-step activation to FdUMP through thymidine kinase and is ~1000-fold more potent than 5-FU. In vivo, FdUrd is rapidly metabolized in the liver and plasma through thymidine phosphorlyase, liberating 5-FU (43, 44). In fact, [3H]5-FU is incorporated into RNA in vitro in HCT116 colorectal cancer cells, and its cytotoxicity is reversed by coadministration of 1 μM uridine (45). These results reflect findings with 5-FU versus folinic acid/5-FU combinations used in the clinic for metastatic colorectal disease, where the latter regimen shows increased response rates without affecting survival (41).

Indeed, we have found no observable toxicity with p.o.-administered OGT 719 in this model, although the cumulative dose of oral OGT 719 administered was 225 times higher than that of i.v. 5-FU (45,000 mg/kg versus 200 mg/kg). Allowing for (approximately) 20% bioavailability of OGT 719 in both rats and mice (data not shown), the mice still tolerated an ~45-fold higher dose of OGT 719 than of 5-FU without any signs of systemic or gastrointestinal toxicity. We have also compared OGT 719 and 5-FU in a rat sarcoma liver metastasis model, where i.v. administered 5-FU showed a marginal therapeutic window because of dose-limiting toxicity, and i.v.- or p.o.-administered OGT 719 was significantly more efficacious.

OGT 719 is now being evaluated in a human clinical trial for primary hepatocellular carcinoma. A bystander mechanism of OGT 719 would suggest that OGT 719 may have broader applications for other tumors that metastasize to the liver, including breast and colorectal carcinoma metastases.

ACKNOWLEDGMENTS

We are grateful to Drs. Chris Moyes and Raj Parekh for helpful comments. We are also grateful to Farid Yafai for technical assistance and to Aston Molecules for assistance on OGT 719 plasma analysis, and we thank Dr. Richard Stockert for the anti-ASGP-R H1 antisemur.

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