Saturable Hepatic Extraction of Gemcitabine Involves Biphasic Uptake Mediated by Nucleoside Transporters Equilibrative Nucleoside Transporter 1 and 2

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Received 30 January 2015; revised 25 April 2015; accepted 27 April 2015

Published online 2 June 2015 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/jps.24498

ABSTRACT: Hepatic arterial infusion (HAI) chemotherapy with gemcitabine (GEM) is expected to be more effective and safer method to treat hepatic metastasis of pancreatic cancer compared with intravenous administration, because it affords higher tumor exposure with lower systemic exposure. Thus, a key issue for dose selection is the saturability of hepatic uptake of GEM. Therefore, we investigated GEM uptake in rat and human isolated hepatocytes. Hepatic GEM uptake involved high- and low-affinity saturable components with K_m values of micromolar and millimolar order, respectively. The uptake was inhibited concentration dependently by *S*-(4-nitrobenzyl)-6-thioinosine (NBMPR) and was sodium-ion-independent, suggesting a contribution of equilibrative nucleoside transporters (ENTs). The concentration dependence of uptake in the presence of 0.1 μM NBMPR showed a single low-affinity binding site. Therefore, the high- and low-affinity sites correspond to ENT1 and ENT2, respectively. Our results indicate hepatic extraction of GEM is predominantly mediated by the low-affinity site (hENT2), and at clinically relevant hepatic concentrations of GEM, hENT2-mediated uptake would not be completely saturated. This is critical for HAI, because saturation of hepatic uptake would result in a marked increase of GEM concentration in the peripheral circulation, abrogating the advantage of HAI over intravenous administration in terms of severe adverse events. © 2015 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 104:3162–3169, 2015

Keywords: transporters; cancer; hepatocytes; equilibrative nucleoside transporter; hepatic transport; kinetics

INTRODUCTION

Pancreatic cancer is a major cause of cancer-related death globally, and has a 5-year survival rate of only about 5% for all stages combined.[1] Surgical treatment offers the best possibility for cure, but pancreatic cancer is likely to become metastatic,[1] especially to the liver (60%).[2] Even patients who receive surgery experience a 50%–80% local recurrence rate and a 25%–50% risk of developing distant metastases,[3] and the 5-year survival rate after recurrence is less than 30%.[4,5] Therefore, postoperative chemotherapy is crucial.

Gemcitabine (2′,2′-difluorodeoxycytidine; GEM) is used as the first-line drug for pancreatic cancer patients,[6] and its dose-limiting toxicity is bone-marrow suppression. GEM is a deoxycytidine nucleotide analog, and is a substrate of nucleoside transporters, that is, equilibrative nucleoside transporters (ENTs; SLC29 family) and concentrative nucleoside transporters (CNTs; SLC28 family),[7–9] which are expressed at the mRNA level in human hepatocytes, although their contribution rates to GEM uptake in normal hepatocytes and cancer cells are still unknown.[10,11] GEM is also used in postoperative chemotherapy. However, chemotherapy is often postponed or the dose reduced in the case of intravenous infusion (i.v.) of GEM owing to the invasiveness of resectional surgery, and in the latter case, the therapeutic effect may be insufficient, especially in patients with hepatic metastasis.[2]

Hepatic arterial infusion (HAI) chemotherapy is a liver-directed treatment option for primary and metastatic hepatic tumor. Liver metastases depend on the hepatic artery for vascularization, whereas normal liver tissues receive blood from both the portal vein and hepatic artery.[12] As HAI enables increased hepatic delivery of drugs, anticancer effectiveness is enhanced. HAI also has the advantage that first-pass hepatic extraction results in a lower systemic concentration and therefore less severe adverse events. For example, HAI of fluorouracil (5-FU), which has a high hepatic extraction rate, achieved a high concentration at hepatocytes together with a low systemic concentration,[13,14] and this resulted in increased overall survival time, increased response rate, and improved physical functioning compared with systemic administration therapy (i.v.) in patients with metastatic colorectal cancer.[15]

Abbreviations used: ENT, equilibrative nucleoside transporter; GEM, gemcitabine; HAI, hepatic arterial infusion; NBMPR, *S*-(4-nitrobenzyl)-6-thioinosine; SLV, standard liver volume; i.v., intravenous infusion; CNT, concentrative nucleoside transporter.

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Journal of Pharmaceutical Sciences, Vol. 104, 3162–3169 (2015)
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As GEM is mainly eliminated by hepatic metabolism (the renal excretion rate of unchanged GEM is less than 10%) and shows a high hepatic extraction rate,[13] it is a good candidate for administration by HAI. Indeed, we and other researchers[16–18] have reported that the systemic concentration of GEM after administration by HAI is lower than that after i.v. administration. We have also reported that administration of 400 mg GEM/body for 30 min by HAI did not lead to severe adverse events in patients with postoperative liver metastases from pancreatic cancer,[19] and we confirmed the effectiveness and safety of GEM HAI.[18–21] The response rate according to the response evaluation criteria in solid tumors was 80%–100%, and severe adverse events, especially leukocytopenia and thrombocytopenia, were not encountered.

The clinical dose of cytotoxic anticancer drugs is determined by the tolerance of the patient to the dose-limiting toxicity (maximal tolerance dose). However, the optimum drug administration schedule for GEM HAI is unclear. This is important, because administration of a dose that resulted in saturation of hepatic extraction would lead to a marked increase of the systemic concentration, which in turn might increase the likelihood of severe adverse events. In other words, to achieve efficacious and safe HAI therapy, dose selection is a critical issue. So, it is necessary to establish in detail the kinetics of hepatic extraction of GEM in hepatocytes, and to predict whether transporters that contribute to GEM uptake are likely to become saturated at clinically relevant concentrations. Therefore, in the present study, we investigated the saturability of hepatic uptake of GEM in rat and human hepatocytes, and established that hepatic extraction of GEM is mediated by high- and low-affinity transporters, ENT1 and ENT2, respectively, of which ENT2 would be the major contributor in the clinical context.

MATERIALS AND METHODS

Chemicals

[^{3}H]GEM (16.3 Ci/mmol) and [^{14}C]inulin (16 mCi/mmol) were purchased from Moravek Biochemicals, Inc. (Brea, California) and American Radiolabeled Chemicals, Inc. (St. Louis, Missouri), respectively. Nonlabeled GEM was purchased from Tokyo Chemical Industry Company, Ltd. (Tokyo, Japan). Collagenase Type I from *Clostridium histolyticum* was obtained from Sigma–Aldrich (St. Louis, Missouri). All other chemicals were commercial products of analytical or cell-culture grade. *S*-(4-nitrobenzyl)-6-thioinosine (NBMPR) was purchased from Wako Pure Chemical Industries, Ltd., (Osaka, Japan).

Animals

Seven- to 10-week-old male Wistar rats (170–200 g) were purchased from Japan SLC, Inc. (Hamamatsu, Japan). Rats were housed under a 12-h light, 12-h dark cycle and were given free access to a normal diet and water. All animal procedures were carried out in accordance with the standards set forth in the Guidelines for the Care and Use of Laboratory Animals at the Takara-machi campus of Kanazawa University.

Isolation of Rat Hepatocytes

Rat hepatocytes were obtained by a collagenase perfusion method with slight modification of published method.[22,23] Briefly, a 7- to 10-week-old rat was anesthetized with diethyl ether and the portal vein was cannulated with an 18G intravenous cannula (Terumo Corporation, Tokyo, Japan) after laparotomy. The liver was perfused with EGTA solution, and the inferior vena cava was cut to remove blood and perfuse EGTA solution. After flushing with 150–200 mL of EGTA solution, the perfusion buffer was changed to 0.05% collagenase solution (150–200 mL). The chest was opened, and the thoracic portion of the inferior vena cava was cannulated via the heart with an 18G intravenous cannula, and the inferior vena cave was ligated. The composition of EGTA solution was 137 mM NaCl, 5.37 mM KCl, 0.42 mM Na_2HPO_4, 0.65 mM NaH_2PO_4, 4.17 mM $NaHCO_3$, 5.55 mM glucose, 0.01% phenol red, 10 mM HEPES, and 0.5 mM EGTA, pH 7.4, and that of collagenase solution was 0.05% collagenase type I, 137 mM NaCl, 5.37 mM KCl, 0.42 mM Na_2HPO_4, 0.65 mM NaH_2PO_4, 5.1 mM $CaCl_2$, 4.17 mM $NaHCO_3$, 5.55 mM glucose, 0.01% phenol red, and 10 mM HEPES, pH 7.4. During perfusion of EGTA or collagenase solution, the flow rate was maintained at 20 mL/min by the use of a tubing pump and the solution was gassed with O_2 and kept at 37°C. Digested liver was transferred to HBSS buffer (135 mM NaCl, 5.0 mM KCl, 0.8 mM Na_2HPO_4, 3.3 mM NaH_2PO_4, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, and 5 mM HEPES, pH 7.4), which was gassed with O_2 at 4°C, and the suspension was filtered through a 150-μm mesh sieve. Isolated hepatocytes were precipitated by centrifugation at 50*g* for 2 min, and the cell pellet was suspended in supplemented HBSS buffer. This suspension was centrifuged again and the cells were suspended at a density of about 1.0×10^6 cell/mL. Uptake tests were conducted as soon as possible, and cell viability was confirmed to be over 80%, using 0.4% (w/v) trypan blue solution. Sodium ion free HBSS buffer (135 mM choline chloride, 5.0 mM KCl, 4.1 mM K_2HPO_4, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, and 5 mM HEPES, pH 7.4) was used instead of HBSS buffer for sodium ion free assay.

Human Hepatocytes

Cryopreserved human hepatocytes derived from a 47-year-old Caucasian female (lot no. HH1009) were purchased from In Vitro ADMET Laboratories Company, Inc. (Columbia, Maryland). The cells were thawed and suspended in Dulbecco's modified Eagle's medium (Life Technologies Corporation, Carlsbad, California) supplemented with 10% fetal bovine serum (Sigma–Aldrich) and penicillin–streptomycin mixed solution [100 U/mL penicillin, 100 μg/mL streptomycin (Nacalai Tesque, Inc., Kyoto, Japan)]. After centrifugation at 50*g* for 2 min, cells were resuspended in HBSS buffer at 4°C and adjusted to a density of about 1.0×10^6 cell/mL. Cells were used for uptake experiments as soon as possible after preparation.

Uptake Study

Aliquots of cell suspension (110 μL) were placed in 1.5 mL tubes on ice. After preincubation at 37°C for 10 min, 110 μL of incubation medium were added to the cell suspension to initiate uptake. The incubation medium was HBSS buffer containing [^{3}H]GEM [rat: 62 nM (37 kBq/mL), human: 123 nM (74 kBq/mL) with/without nonlabeled GEM] and was kept at 37°C. These concentrations of [^{3}H]GEM were chosen on the basis of preliminary experiments with rat and human hepatocytes (data not shown). At designated times, 200 μL aliquots of the mixture were placed in centrifuge tubes containing 50 μL of 3 M KOH, and covered with a layer of 100 μL of mixed oil (density = 1.027–1.032 g/cm^3) consisting of silicon oil

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