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Synthesis, Crystallization, and Biological Evaluation of an Orally Active Prodrug of Gemcitabine

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Abstract: The design, synthesis, and biological characterization of an orally active prodrug (**3**) of gemcitabine are described. Additionally, the identification of a novel co-crystal solid form of the compound is presented. Valproate amide **3** is orally bioavailable and releases gemcitabine into the systemic circulation after passing through the intestinal mucosa. The compound has entered clinical trials and is being evaluated as a potential new anticancer agent.

Gemcitabine (**1**, Figure 1) (2′,2′-difluorodeoxycytidine, dFdC[a]) is a pyrimidine nucleoside analogue with proven anticancer efficacy for a variety of solid tumor types including pancreatic, NSCLC, and breast cancer.[1] Gemcitabine HCl is marketed as Gemzar in more than 70 countries and is administered as a parenteral formulation with a 30 min intravenous infusion of 1000−1250 mg/m^2 given once weekly for a 3- or 4-week cycle. Gemcitabine is activated within cells by sequential phosphorylation by deoxycytidine kinase to gemcitabine mono-, di-, and triphosphate nucleotides.[2] Alternatively, gemcitabine may be deaminated to its inactive uridine metabolite 2′,2′-difluorodeoxyuridine (dFdU) by deoxycytidine deaminase, which is present at high levels in both human plasma and liver[3] (Figure 2). Deamination occurs rapidly in plasma with a half-life of approximately 70 min. Despite this rapid inactivation, gemcitabine is clinically efficacious in a number of solid tumors.

Several clinical studies have indicated that the antitumor effect of gemcitabine is schedule dependent and that lower doses are efficacious.[4] Experiments have been performed to study the toxicity associated with a single oral dose of gemcitabine in CD-1 mice using doses approximating clinically efficacious doses.[5]

While high-dose oral gemcitabine was associated with expected hematotoxicity, gastrointestinal toxicity, which is consistent with local rather than systemic effects, also was observed. This precludes the use of oral gemcitabine in a clinical setting.

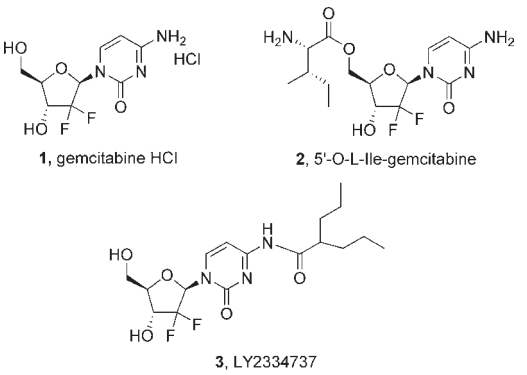

Figure 1. Chemical structures of gemcitabine and prodrugs of gemcitabine.

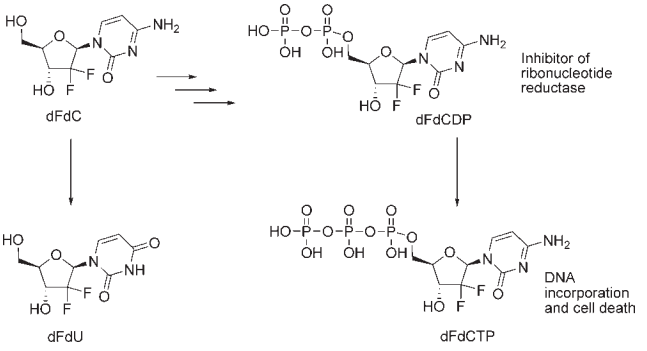

Figure 2. Activation and inactivation pathways for gemcitabine.

Prodrugs have proven to be an effective method for the oral delivery of several nucleoside drugs such as capecitabine,[6] a carbamate prodrug of 5-fluorouracil (5-FU), and valacyclovir, the L-valine ester of acyclovir.[7] Modification of acyclovir to valacyclovir resulted in a 5-fold increase in oral bioavailability via recognition and active transport by the amino acid transporter PEPT-1. Recently, studies of gemcitabine prodrugs have appeared in the literature. Amidon et al. reported the synthesis and evaluation of a series of amino acid ester prodrugs of gemcitabine, targeting the PEPT-1 transporter.[8] The L-isoleucine derivative (**2**) was found to be relatively stable to hydrolysis in pH 7.4 buffer and exhibited a very high degree of stability when incubated with crude cytidine deaminase. Interestingly, **2** was found to be approximately 10-fold more stable in Caco-2 homogenates than the L-valine analogue, with an estimated half-life of 75 min.

As part of our effort to identify an orally active prodrug of gemcitabine, we chose to introduce the prodrug moiety at the N^4-position on the cytidine ring. The amide linkage was postulated to be more stable to both chemical and enzymatic hydrolysis relative to the corresponding ester derivatives. Furthermore, functionalization of the cytidine amine was proposed to improve bioavailability by blocking the site of deamination to dFdU, thus reducing first-pass metabolism. In our earlier work, amino acid amide derivatives of gemcitabine were prepared with the goal of achieving the desired chemical and enzymatic stability while maintaining affinity for PEPT-1.[9] However, under near neutral or basic conditions, rapid rearrangement to the corresponding carboxamide

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[a] Abbreviations: dFdC, 2′,2′-difluorodeoxycitidine; NSCLC, non-small-cell lung cancer; dFdU, 2′,2′-difluorodeoxyuridine; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; HOBt, 1-hydroxybenzotriazole; DIEA, *N*,*N*-diisopropylethylamine; NMM, *N*-methylmorpholine; DCM, dichloromethane; ppy, 4-piperidinopyridine; TFA, trifluoroacetic acid; TMSCl, trimethylsilyl chloride; TSA, *p*-toluenesulfonic acid; SIH, small intestine homogenate.

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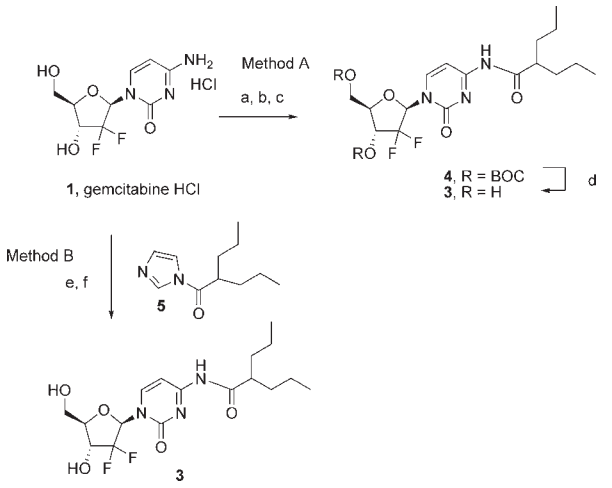
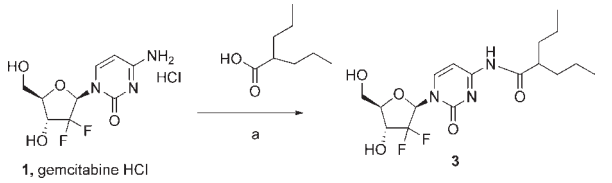
Scheme 1[a]



[a] Reaction conditions: (a) BOC_2O (excess), 1 M KOH/dioxane (1:1); (b) (i) BOC_2O, dioxane; (ii) 1 M KOH; (c) valproic acid, EDC, HOBt, ppy, DIEA, DCM; (d) TFA, DCM; (e) (i) TMSCl, pyridine, 0 °C; (ii) **5**, MeCN, 45 °C; (f) (i) EtOH; (ii) H_2O, 45 °C.

Scheme 2[a]



[a] Reaction conditions: (a) EDC, HOBt, NMM, DMF/DMSO (3:1), 55 °C.

occurred, generating a species that could not be converted to the desired parent compound. In order to further probe the utility of this type of moiety, a series of additional amide prodrugs were prepared, culminating in the discovery of valproate amide **3**.

The synthesis of **3** is shown in Scheme 1. Gemcitabine hydrochloride was protected as the 3′,5′-bis-*tert*-butoxycarbonyl derivative by reaction with di-*tert*-butyldicarbonate in KOH/dioxane as previously reported by Gallo.[10] The valproic acid moiety was introduced utilizing an EDC based coupling to produce **4**, which was subsequently deprotected with trifluoroacetic acid to give **3**. Alternatively, gemcitabine hydrochloride was protected in situ as the 3′,5′-*O*-bis-silyl ether by reaction with chlorotrimethylsilane in anhydrous pyridine. This solution was treated with an acetonitrile solution of activated ester **5**, prepared by reaction of valproic acid with 1,1-carbonyldiimidazole. The silyl groups were subsequently hydrolyzed under aqueous conditions to give **3** in 96% yield . An alternative route to **3** was eventually developed that completely avoided the use of protecting groups (Scheme 2). Valproic acid was coupled directly to gemcitabine using peptide coupling conditions (EDC/HOBt/NMM) to give **3** in excellent yield (95%).

As a free base, **3** was not crystalline. Therefore, to provide a means of purification (via crystallization) and oral drug delivery, a screen for salts and co-crystals of **3** was conducted. The crystallization screen yielded viable salts with *p*-toluenesulfonic acid (TSA) and benzenesulfonic acid. Ultimately, a novel TSA salt co-crystal (**6**) comprising 2 equiv of **3**, 1 equiv of TSA, and 1 equiv of water was chosen as the crystalline form

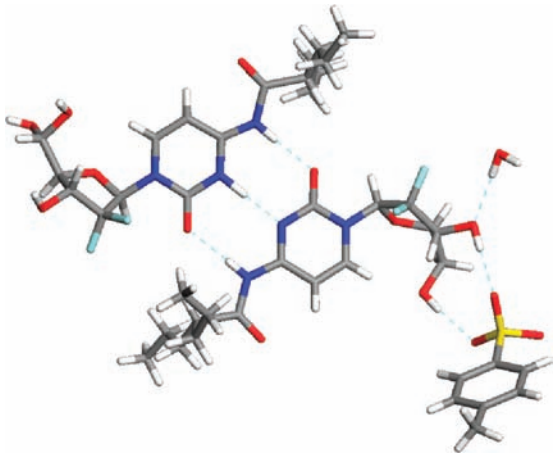


Figure 3. X-ray structure of the 2:1:1 gemcitabine prodrug hemitosylate hemihydrate **6**.

Table 1. Solution Stability and Solubility of **3** in Buffer Solutions at 40 °C for 4 h[a]

pH	% hydrolysis to gemcitabine	solubility (mg/mL)
1	21	1.12
2	12	1.79
4	2	> 2.0
6	0	> 2.0
8	0	> 2.0

[a] 100 μg/mL of **3** was dissolved in each buffer solution and incubated at 40 °C for 4 h.

for future development based on its superior physicochemical properties and ease of preparation. X-ray crystal structure analysis of the salt co-crystal confirmed a single proton transfer between TSA and 1 equiv of **3** (Figure 3). Protonation of the cytosine ring creates a complementary structure to the neutral molecule, resulting in a second equivalent of **3** being sequestered in the crystal structure by triple hydrogen bonding like that observed for the guanine−cytosine (GC) base pair in DNA. Interestingly, hydrogen bonding in the cytosine−cytosinium ion dimer appears to be stronger (based on shorter intermolecular contacts) than in the GC base pair.[11]

To deliver the intact prodrug to the systemic circulation, the compound must first be stable at the various pH environments encountered in the gastrointestinal tract following oral dosing. The solution stability of the compounds was studied in a series of buffer solutions ranging from pH 1 to pH 8 (Table 1). The stability of **3** was found to be pH dependent across the entire pH range. Approximately 21% degradation (hydrolysis to gemcitabine) occurred at pH 1 and none at neutral pH 6−8 after 4 h at 40 °C.

To assess the enzymatic stability of the prodrugs, the rate of hydrolysis was determined in small intestine homogenates (SIH). Crude homogenates of small intestinal epithelial cells were prepared from sections of the upper small intestine from CD-1 mice and humans. The rate of hydrolysis of **3** (at 96 μM) in SIH incubations (2.2−4.4 mg/mL protein) was slow (< 10 (pmol/mg)/min) in both species. In comparison, turnover of the 5′-*O*-isoleucine ester **2** was found to be substantially more rapid at 2.3 and 3.7 (nmol/mg)/min in mouse and human, respectively. Over a 30 min incubation period, these rates correspond to 100% hydrolysis of the *O*-Ile ester **2**, compared to approximately 1% of the prodrug **3**. The *in vitro* hydrolysis profile of the compound was further evaluated in liver S9

Table 2. Comparison of Efficacy of **3** to Gemcitabine (**1**) When Dosed Orally in a Human Colon HCT-116 Xenograft Model

dose[a] (mg/kg)	gemcitabine free base equivalent dose (mg/kg)	route and schedule	maximum tumor reduction[b] (% vehicle)
3.77 (**3**)	2.5	oral, qd × 14	56.3 ± 6.0
7.55 (**3**)	5.1	oral, qd × 14	67.1 ± 4.9
160 (**1**)	140	ip, q3d × 4	71.5 ± 3.8

[a] Mice (10 animals per group) were treated with the vehicle or the indicated drug. LY2334737 was dosed by oral gavage daily for 14 days (qd × 14). Alternatively, gemcitabine was administered intraperitoneally every 3 days for a total of 4 doses (Q3D × 4). Tumor volumes and animal weights were measured 3 times a week for 6 weeks. One mouse out of 10 died in the 7.55 mg/kg **3** group. [b] Maximum reduction in tumor volume observed during the drug treatment period versus the vehicle treatment group. Statistically significantly different ($p < 0.0001$) from the vehicle control group.

incubations to determine the extent of hepatic activation. The hydrolysis rate in hepatic S9 (100 μM) was also relatively slow, at 27 (pmol/min)/mg in human S9 and 11 (pmol/mg)/min in mouse. These results suggest that **3** would be orally absorbed as the intact prodrug, with minimal hydrolysis occurring in the gastrointestinal tract due to the molecule's chemical and enzymatic stability. Slow hydrolysis in the liver, however, should result in sustained delivery of gemcitabine to the systemic circulation.

Compound **3** was administered orally to CD-1 mice at a dose of 14.3 mg/kg, and systemic plasma concentrations of both **3** and gemcitabine were analyzed. A single sample was also collected from the hepatic portal vein at 5 min postdose. The prodrug was rapidly absorbed and passed the intestinal epithelium largely intact, with a mean concentration of 653 ng/mL in portal blood at 5 min. The mean gemcitabine concentration was 99 ng/mL in the same samples, suggesting that a large percentage of the absorbed fraction is stable during absorption. Systemic plasma samples collected at 5 min postdose had approximately 3-fold lower concentrations of **3** compared to the corresponding portal sample, consistent with hepatic first pass hydrolysis. Systemic exposure (AUC) to gemcitabine was 778 ng·h/mL, and C_{max} was 373 ng/mL. These values compare to 536 ng·h/mL and 535 ng/mL in mice dosed with a molar equivalent dose of oral gemcitabine. The T_{max} values were 1 h for **3** and 0.5 h for gemcitabine. Oral dosing of the prodrug also resulted in prolonged gemcitabine exposure and a change in the shape of the gemcitabine concentration−time profile, suggesting formation-rate-limited kinetics.

In a separate study, compound **3** was administered orally to CD-1 mice at doses of 6, 12, and 24 mg/kg and concentrations of **3** and gemcitabine were measured, as well as the concentration of dFdU produced via subsequent deamination of gemcitabine. The ratio of dFdU to gemcitabine was found to be less than half of that observed in mice who received equivalent doses of gemcitabine alone, which provides evidence for a reduction in first-pass metabolism through blocking of the site of deamination with the prodrug moiety.

The *in vivo* antitumor activity of **3** was evaluated in a HCT-116 human colon tumor xenograft using CD-1 nude mice (Table 2). Initial in vivo 3-day cytotoxicity studies indicated that the prodrug itself was not cytotoxic to HCT-116 cells and therefore needed to be cleaved to release gemcitabine (data not shown). The compound displayed significant antitumor activity at the 7.55 and 3.77 mg/kg doses when given daily for 14 days. These data also indicated that the 7.55 mg/kg dose of the prodrug was equally efficacious to gemcitabine given intraperitoneally at 160 mg/kg for a total of four doses. The treatment was well tolerated with 2% or less weight loss observed for all groups.

In summary, an orally active prodrug of gemcitabine was developed that possesses excellent chemical and enzymatic stability and delivers intact prodrug to the systemic circulation, thereby reducing instestinal exposure to gemcitabine. The compound achieved oral efficacy on a daily schedule that is equivalent to a clinically relevant ip dose of gemcitabine in HCT-116 human colon cancer xenografts. On the basis of these results, **3** was selected for further development and has advanced into phase I clinical trials.

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Supporting Information Available: Detailed experimental procedures for the synthesis, characterizationm and crystallization of compound **3**. This material is available free of charge via the Internet at http://pubs.acs.org.

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