Nucleoside Based Self-Assembling Drugs for Localized Drug Delivery

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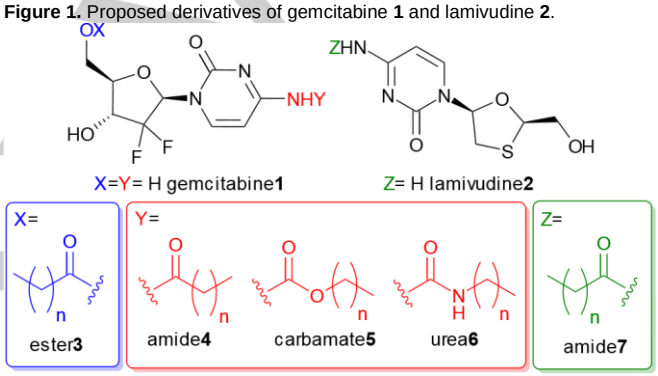
Abstract: We have synthesized a range of gelators based on nucleoside analogues gemcitabine and lamivudine, characterizing representative gels from the series using rheology and TEM. Growth inhibition studies of gemcitabine derivatives confirmed the feasibility of these compounds as novel treatments, indicating the potential of nucleoside based gelators for localized drug delivery.

Defined as structures possessing "a continuous microscopic structure with macroscopic dimensions that are permanent and solid-like in rheological behaviour despite being derived from systems that were mostly liquid",[1] gels are a diverse class of multi-component molecular systems. In the simplest terms, they contain both liquid and a solid 3D matrix.

Low molecular weight gels (LMWGs) are formed by the self-assembly of low molecular mass molecules (≤ 3000 gmol^{-1}), and contain small amounts (typically < 2 *wt%*) of gelator in combination with an organic and/or aqueous liquid.[2] The gelator self-assembles in the liquid to form a continuous phase governed by non-covalent interactions such as π-stacking, hydrogen bonding and dispersion forces.[3] These reversible interactions make LMWGs attractive agents for a range of applications including drug delivery, tissue engineering, catalysis, green chemistry, chemical sensors and electronics, as they can be designed to gelate under very specific and/or mild conditions.[4, 5, 6] LMWGs therefore offer immense potential for novel, highly-specific drug delivery systems *via*: 1) drug encapsulation with diffusion and/or degradation release,[7] 2) covalent binding of drug molecule(s) released by bond cleavage upon interaction with target stimulus,[8] or 3) prodrug gelator compounds that form active systems when self-assembled (therapeutic molecular gels).[9, 10, 11, 12, 13]

With LMWG drug delivery systems, the ability of gels to remain localized *in vivo* allows for greater site-specific delivery and can vastly increase drug efficacy while minimising adverse systemic toxicity. We proposed to synthesise a library of potential gelator derivatives of gemcitabine **1** and lamivudine **2** as shown in **Figure 1**. We hypothesized that a LMWG derived from chemotherapeutic gemcitabine **1** would allow for the development of an intra-tumoral therapy, potentially enhancing *in vivo* drug activity. Simultaneously, this approach could hinder the first-pass metabolism of gemcitabine by cytidine deaminase (CDA) and deoxycytidine deaminase (dCDA). We also believed a LMWG derived from a HIV antiretroviral lamivudine **2** would allow for the development of a topical pre-exposure treatment applied vaginally to protect the user from HIV infections. Vaginal drug delivery has been highlighted as an important tool in the fight against HIV and AIDS,[14] with studies such as the CAPRISA 004 Tenofovir gel trial indicating that prevention of male-to-female sexual transmission of HIV-1 is achievable using this approach.[15]

Figure 1. Proposed derivatives of gemcitabine **1** and lamivudine **2**.



Most importantly, the Tenofovir gel is a simple cellulose-based aqueous gel and retention in the vagina is often poor. Hence, there is clearly a need for new and innovative approaches to vaginal gel formulation that addresses this deficiency.

In order for a system to successfully form gels a balance of non-covalent interactions and solubility is required, illustrated by the fact that gelators are usually only able to form gels in a narrow range of solvents.[16] The gemcitabine **1** core contains multiple hydrogen bond donor and acceptor groups and a pyrimidine base allowing for π-stacking that we believed would facilitate creation of a self-assembled network; introduction of alkyl groups to the system would decrease aqueous solubility and create the solvophobic forces necessary for gelation under aqueous conditions.[3, 4] Ester prodrugs are commonly synthesized in order to enhance lipophilicity, increasing passive membrane permeability of water soluble drugs.[17] The ester moiety is also readily hydrolysed under physiological conditions, and multiple examples of efficacious gemcitabine-ester prodrugs have been reported.[18, 19, 20] We decided to synthesise ester derivatives of the 5'-hydroxyl group (**Table 1**, entries 1-5) as previous studies have shown the importance of the 2'-hydroxyl group for gelation of the inert analogue 2'-deoxycytidine.[21] Acylation of the 5'-hydroxyl group could also be achieved in one pot from gemcitabine **1** using enzymatic esterification.[22] Chain lengths ranging from tetradecanoyl (**2a**) to hexanoyl (**2e**) were chosen to provide varying degrees of hydrophobicity.

By blocking the site of potential deamination, modification of the 4-amino position affords the opportunity to increase both

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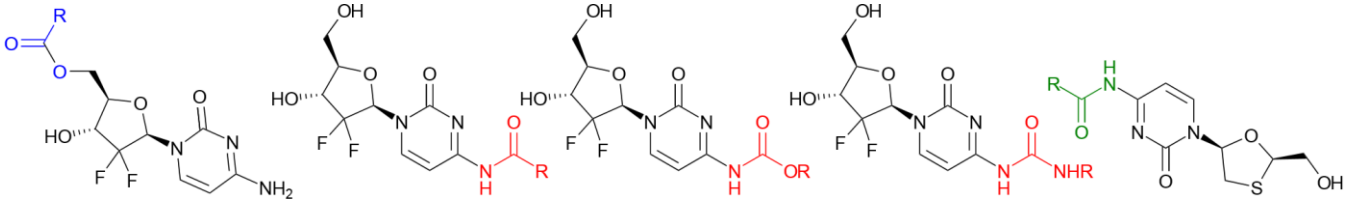
Table 1. Synthesized Derivatives of Lamivudine **2** and Gemcitabine **1**.



Entry		R	%Yield[a]	%Purity[b]	cLogP[c]	LogS$_w$[c]	Entry		R	%Yield[a]	%Purity[b]	cLogP[c]	LogS$_w$[c]
1	3a	$(CH_2)_{12}CH_3$	89	99	5.88	-4.78	11	5a	$(CH_2)_5CH_3$	28	97	2.36	-2.83
2	3b	$(CH_2)_{10}CH_3$	77	98	4.82	-4.55	12	5b	$(CH_2)_3CH_3$	29	95	1.30	-2.00
3	3c	$(CH_2)_8CH_3$	66	98	3.77	-4.03	13	5c	$CH_2CH(CH_3)CH_3$	26	95	1.17	-1.83
4	3d	$(CH_2)_6CH_3$	95	99	2.71	-3.20	14	6a	$(CH_2)_{13}CH_3$	55	96	5.38	-5.10
5	3e	$(CH_2)_4CH_3$	70	>99	1.65	-2.37	15	6b	$(CH_2)_{11}CH_3$	55	98	4.32	-4.28
6	4a	$(CH_2)_{12}CH_3$	55	98	5.80	-5.77	16	6c	$(CH_2)_7CH_3$	53	96	3.26	-3.45
7	4b	$(CH_2)_{10}CH_3$	23	99	4.74	-5.19	17	6d	$(CH_2)_5CH_3$	36	97	2.21	-2.62
8	4c	$(CH_2)_8CH_3$	41	99	3.68	-4.59	18	6e	$(CH_2)_3CH_3$	26	96	1.15	-1.79
9	4d	$(CH_2)_6CH_3$	42	99	2.62	-3.97	19	7a	$(CH_2)_6CH_3$	56	>99	1.87	-3.00
10	4e	$(CH_2)_4CH_3$	43	99	1.56	-3.33	20	7b	$(CH_2)_8CH_3$	31	99	2.93	-3.83
							21	7c	$(CH_2)_{10}CH_3$	44	>99	3.98	-4.66

[a] Yield of purified compound isolated after flash column chromatography. [b] Determined by RP-HPLC on a Phenomenex Luna C_8 column. [c] Calculated using ACD/Labs software.

chemical and enzymatic stability of gemcitabine **1**. Amide, carbamate and urea derivatives at the 4-amino position were therefore also chosen (**Table 1**, entries 6-10, 11-13 and 14-18 respectively) as their greater enzymatic stability generally allows for increased half-lives over those of their respective esters. We postulated that the increased stability to hydrolysis of these derivatives may potentially allow for lower doses and a prolonged therapeutic effect. Indeed, examples of gemcitabine *N*-amides have been shown to exhibit cytotoxicity *in vivo* and increased metabolic stability,[23, 24] with 4-*N*-alkanoyl gemcitabine derivatives, similar to those synthesised here showing similar *in vitro* cell growth inhibition with IC_{50} values in the low nanomolar range and comparable to the parent compound.[25] Also 4-*N*-acylated squalene derivatives of gemcitabine self-assembled in water into nano assemblies that showed superior anticancer properties in a preclinical leukeamia model.[26] In a similar manner vitamin E conjugated to gemcitabine via the *N*4-amino group also formed nano assemblies that showed increased growth inhibition against a pancreatic cell line.[27] Amide, carbamate and urea functional groups are also known to promote gelation.[2, 3, 4, 21]

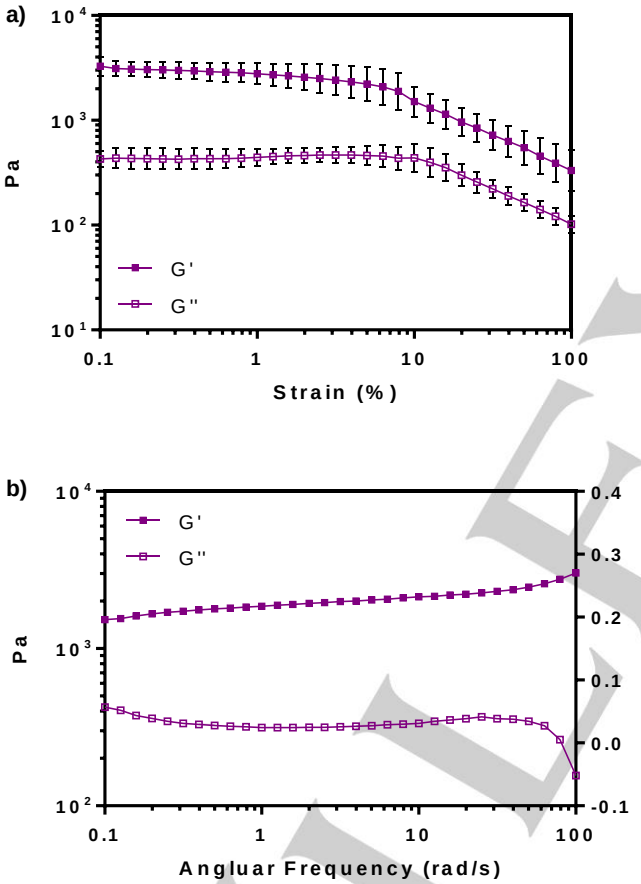
Lamivudine **2** has both primary amine and primary alcohol functionalities that can be readily derivatized. Modification of the alcohol would leave only 1 hydrogen-bond donor in the resultant derivatives, which we believed would decrease the potential for creation of a self-assembled gelator network based on our previous studies.[3, 28] We therefore decided to modify the amino group to produce amide derivatives **7** of varying chain lengths (**Table 1**, entries 19-21). Prodrugs have been shown to be active when administered vaginally; in addition, the increased lipophilicity has the potential to increase drug absorption into the vaginal epithelium.[14]

The gemcitabine and lamivudine derivatives were synthesized using known procedures (see Supporting Information)[19] and were isolated in high purity (≥ 95%) in moderate to excellent yields (**Table 1**). Owing to the relatively high cLogP values of compounds in each series (**Table 1**), gelation was approached using an 'anti-solvent' system;[29] solubilisation of the more lipophilic compounds in an organic solvent prior to the addition of water was thought to offer the greatest opportunity for successful gelation. The derivatives were initially dissolved in ethanol at 60°C; water pre-heated to 60°C was then added to each solution to achieve a final total volume of 500 µL and a final concentration of 0.5 *wt%* at various solvent volume fractions (Φ_{EtOH}). The samples were then allowed to cool to room temperature for 18 h before stability to inversion was investigated (**Figure S1** Supporting Information). The gemcitabine and lamivudine derivatives displaying the most promising gel-like behaviour in stability to inversion screening (amides **4d** and **7b**) were analysed using TEM (**Figure S2** Supporting Information). The majority of structures observed in both gels were found to be moderately cross-linked, with morphologies ranging from fibres to ribbons. Fibre diameters were found to range from 1-40 nm with lengths in the range of hundreds of microns. Lamivudine *N*-amide **7b** exhibited a less densely packed network of fibres to that of **4d**.

Oscillatory rheology tests were used to characterise the strength of gel **4d** (Φ_{EtOH} 0.05), chosen as representing the best from both series. Amplitude sweeps carried out using variable strain and constant frequency at 37°C showed storage modulus G' was consistently greater than loss modulus G" (**Figure 2a**), suggesting that some cross-linking interactions between nanofibres remained intact; G' was also comparable to that of gels used in drug delivery applications, with values typically reported in the range of 10^3 Pa.[11, 13, 30] Frequency sweeps were carried out

at 37°C under constant strain as determined by the centre of the linear viscoelastic region from the amplitude sweep (**Figure 2b**). The sample showed G' values greater then G", however the difference is less than the order of magnitude expected for a highly cross-linked nanofibrillar structure.[31] The loss factor tan(δ) was calculated to be 0.20 – 0.30 and is suggestive of a cross-linked network with few stabilising linkages between morphologies.[32] These measurements provide insight as to how the gels may behave when administered *via* injection intra-tumourally (our targetted application for gemcitabine derivatives). This approach has been previoulsy reviewed for polymer gels by Wolinsky *et al*.[33] and progressed to porcine preclinical studies for Oncogel® - poly(lactide-co-glycolide) and poly(ethylene glycol) tri-block copolymer with paclitaxel.[34] Whereas we envisage the lamivudine derivatives to be applied topicallly.

Figure 2. Rheological measurements for 4d Φ_{EtOH} 0.05 (0.5 *wt%*).



[a] Amplitude sweep carried out γ = 0.1 – 100%, ω = 10 rad s^{-1}, T= 37°C; [b] Frequency sweep carried out ω = 0.1 – 100 rad s^{-1}, γ = 1%, T= 37°C, standard deviation (G') 2040 ± 620 Pa. In all cases figures are examples of n = 4.

To investigate *in vitro* antitumor growth inhibitory properties of **4d** LMWGs, the (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric cell proliferation assay was used; activities were compared with that of other derivatives displaying gel-like behaviour and the parent compound. As gemcitabine is a first-line treatment for pancreatic and used to treat gastric cancer, compounds **4d**, **5a** and **6c** along with gemcitabine **1** were screened against the human-derived MIA PaCa-2 (pancreatic adenocarcinoma) and MKN-7 (gastric adenocarcinoma) cell lines to determine test agent concentrations that inhibited cell growth by 50% (GI_{50}). To confirm the selectivity of the compounds towards these cancer cell lines an additional non-transformed cell line was employed (MRC-5 foetal lung fibroblast). In the MIA PaCa-2 cell line the GI_{50} value of gemcitabine **1** was found to be 3.02 ± 1.81 nM (**Table 2**, entry 1). Of the other tested compounds **4d** was found to have the highest activity, yet displayed a 10-fold decrease in growth inhibitory activity compared to the parent compound (**Table 2**, entry 2). Compounds **5a** and **6c** both displayed GI_{50} values in the micromolar range (**Table 2**, entries 3 and 4), a result consistent with recent findings.[35] A similar trend was observed in the MKN-7 cells; gemcitabine **1** demonstrated a GI_{50} of 13.52 ± 9.66 nM (**Table 2**, entry 1), with compound **4d** again displaying the highest activity of the gemcitabine derivatives synthesized exhibiting a GI_{50} of 70.85 ± 34.26 nM (**Table 2**, entry 2). In the literature other 4-*N*-alkanoyl gemcitabine derivatives show similar cell growth inhibition to the parent compound. In contrast, the lower potency observed for **4d** could be attributed to its shorter carbon chain length, and hence reduced lipophlicity required to drive passive diffusion i.e C8 as compared to 4-*N*-alkanoyl gemcitabine derivatives with C9 to C13 chains lengths as evaluated by Pulido *et al.*[25]

Table 2. Solution phase growth inhibition studies for gemcitabine 1 and conjugates 3d, 4a and 5c in MIA PaCa-2, MKN-7 and MRC-5 cells.

Entry		GI_{50} (nM)[a]		
		MIA PaCa-2	MKN-7	MRC-5
1	**1**	3.02 ± 1.81	13.52 ± 9.66	> 10000
2	**4d**	34.91 ± 13.79	70.85 ± 34.26	2000 ± 560
3	**5a**	1430 ± 760	786 ± 100	> 10000
4	**6c**	840 ± 460	670 ± 450	> 10000

[a] GI_{50} represents the concentration at which the test agent inhibits cell growth by 50%. Assays were carried out in n = 3.

Screening with the MRC-5 fibroblasts demonstrated the excellent selectivity of gemcitabine and its analogues towards the carcinoma cell lines; a 1000-fold decrease in potency was demonstrated for gemcitabine **1**, carbamate **5a** and urea **6c** (**Table 2**, entries 1, 3 and 4) against MRC5 fibroblasts and a 200-fold decrease was observed for **4d** (**Table 2**, entry 2). Whilst the selectivity cannot be qualified without further testing, these results support the hypothesis that these gelating entities could be potential therapies for localized cancer drug delivery.

In conclusion, a range of gemcitabine and lamivudine derivatives have been synthesized and their ability to form gels investigated. Gemcitabine ester series **3** proved to all be generally poor gelator agents, likely due to steric hindrance introduced by the position of the acyl chain precluding the molecules from forming self-assembled networks. Initial screening of the gemcitabine amide **4**, carbamate **5** and urea **6** series along with the lamivudine amide **7** series highlighted a range of promising gel combinations involving compounds **4d**, **5a**, **6c** and **7b**. TEM

studies showed gels **4d** and **7b** to be moderately cross-linked fibrous networks of varying morphologies. Solution phase growth inhibition studies of the gemcitabine derivatives **4d**, **5a** and **6c** highlighted amide derivative **4d** as displaying the most potent activity towards MIA PaCa-2 and MKN-7 carcinoma cell lines with nanomolar potency. Further rheology studies for the gemcitabine derivative **4d** gels confirmed the viscoelastic nature. These results demonstrate that gemcitabine amide derivative **4d** is suitable for further investigation as a chemotherapeutic agent and nucleoside based LMWG derivatives are suitable for further progression in localized drug delivery applications including vaginal drug delivery.

Acknowledgements

Support from Mike Fay at the Nottingham Nanotechnology and Nanoscience Centre (NNNC) for the TEM Imaging is gratefully acknowledged. We are also grateful to EPSRC (EP/I01375X/1) and AstraZeneca for funding.

Keywords: drug delivery • gels • gemcitabine • lamivudine • nucleosides

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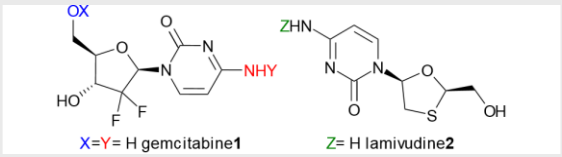

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Title

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