Nucleoside Based Self-Assembling Drugs for Localized Drug Delivery


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. 4-6 LMWGs therefore offer immense potential for novel, highly-specific drug delivery systems via: 1) drug encapsulation with diffusion and/or degradation release; 2) covalent binding of drug molecule(s) released by bond cleavage upon interaction with target stimulus; or 3) prodruk gelator compounds that form active systems when self-assembled (therapeutic molecular gels). 7, 8, 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 deoxyctydine 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, which 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 gemicitabine-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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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-alkylamino gemcitabine derivatives, similar to those synthesised here showing similar in vitro cell growth inhibition with IC50 values in the low nanomolar range and comparable to the parent compound.25 Also 4-N-acetylated squalene derivatives of gemcitabine self-assembled in water into nano assemblies that showed superior anticancer properties in a preclinical leukemia model.26 In a similar manner vitamin E conjugated to gemcitabine via the N4-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

The gemcitabine and lamivudine derivatives were synthesized using known procedures (see Supporting Information)23 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 (Φw). 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 (θHIEC 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 10³ Pa.31,19,30 Frequency sweeps were carried out

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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.\(^3\) The loss factor tan(θ) was calculated to be 0.20 – 0.30 and is suggestive of a cross-linked network with few stabilising linkages between morphologies.\(^3\) These measurements provide insight as to how the gels may behave when administered via injection intra-tumorally (our targeted application for gemcitabine derivatives). This approach has been previously reviewed for polymer gels by Wolinsky et al.\(^3\) and progressed to porcine preclinical studies for Oncogel® (poly(lactide-co-glycolide) and poly(ethylene glycol) tri-block copolymer with paclitaxel).\(^4\) Whereas we envisage the lamivudine derivatives to be applied topically.

Figure 2. Rheological measurements for 4d Φ = 0.05 (0.5 wt%).

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.\(^5\) 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 lipophilicity 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.\(^6\)

<table>
<thead>
<tr>
<th>Entry</th>
<th>MIA PaCa-2</th>
<th>MKN-7</th>
<th>MRC-5</th>
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<tbody>
<tr>
<td>1</td>
<td>3.02 ± 1.81</td>
<td>13.52 ± 9.66</td>
<td>&gt; 10000</td>
</tr>
<tr>
<td>2</td>
<td>34.91 ± 13.79</td>
<td>70.85 ± 34.26</td>
<td>2000 ± 560</td>
</tr>
<tr>
<td>3</td>
<td>1430 ± 760</td>
<td>786 ± 100</td>
<td>&gt; 10000</td>
</tr>
<tr>
<td>4</td>
<td>840 ± 460</td>
<td>670 ± 450</td>
<td>&gt; 10000</td>
</tr>
</tbody>
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[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.

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Low molecular weight gelators based on nucleoside analogues gemcitabine and lamivudine were synthesized and shown to offer great potential for localized drug delivery systems.
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