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In mammalian cells, the cation-independent mannose 6-phosphate/insulin-like growth factor II (M6P/IGFII) and cation-dependent mannose 6-phosphate (CD-MPR) receptors, have been identified as pivotal targets that modulate cellular response because of their role in protein trafficking. Both these receptors are functionally complimentary and can partially compensate for the absence of the other. These sorting receptors play an important role of transporting M6P-bearing glycoproteins from the trans-Golgi network (TGN) to lysosomes mediated through their M6P binding sites. Both receptors transport important enzymes to the intracellular acidic pre-lysosomal compartments where low pH leads to the release of the enzymes from the complex. The receptor then gets recycled into the Golgi apparatus. However, only the M6P/IGFII receptor is anchored to the cell surface membrane and has been implicated in the internalisation of M6P bearing compounds. Importantly, it modulates the activity of a variety of intracellular acidic pre-lysosomal compartments including latent transforming growth factor-β (LTGFβ) precursor, urokinase-type plasminogen activator receptor, glycoprotein D of the herpes virus, granzyme B an essential factor for T cell-mediated apoptosis and proliferin. This has resulted in an enormous interest in the design of M6P bearing compounds that target the M6P/IGFII receptor as it offers an efficient means for internalisation of high specificity therapeutics. This approach has been used to deliver therapeutic compounds in enzyme replacement therapies in lysosomal diseases like Fabry disease, aid wound healing, as a treatment for breast cancer, and to combat viral infections. However, the approach suffers a major drawback as the phosphomonoester bond of M6P is prone to hydrolysis by various phosphatase enzymes. This dramatically reduces its binding efficiency to the receptor thereby compromising its potency. This problem has been circumvented by the design of several isosteric M6P analogues with phosphonate, carboxylate or malonate groups, which have higher affinity to the receptor and a stronger stability in human serum than M6P.

This approach is successful in overcoming the issues with hydrolysis of the phosphomonoester bond, yet falls short as these analogues can only target the receptors present on the cell surface. In the steady state, ~90 % of the M6P/IGFII receptors are localised in the transmembrane compartments while the remainder stays on the cell surface. The receptor has a relatively long half-life (~20 hours) and recycles between the trans-Golgi network, endosomes and the plasma membrane. In this communication, we report a novel approach to improve ligand-receptor protein interaction in cells whilst overcoming stability issues associated with M6P. We demonstrate this by exploring a prodrug (analogue 2) that undergoes intracellular chemical modification by esterases to yield an active M6P analogue (analogue 1) (Figure 1a, see Supporting Information for chemical synthesis and characterisation; section S8.1, S9.1 and Figure S1-11), resulting in a sustained and focused therapeutic strategy in an in vitro model of wound healing.

![Figure 1](image-url)
The design of phosphonate analogue 1 is based on established principles of bioisosteric M6P analogues by replacing the P-O bond at C6 by a methylene bridge. Moreover, the replacement of the hydroxyl group at the anomic position by an aromatic substituent slightly improves recognition by the M6P/IGFII receptor. This could be due to the hydrophobic interactions between the aromatic moiety of analogue 1 and the binding pocket of the M6P/IGFII receptor. Previous studies have demonstrated that neutral ester prodrugs are relatively benign towards enzymatic degradation, thereby altering their apparent elimination and half-life. Hence analogue 2 was designed by masking analogue 1 via esterification of the phosphate group to yield a non-charged bis(pivaloyloxyethyl) (POM) derivative. Importantly, derivatisation of phosphates decreases the polarity of the parent drug thereby promoting its cellular internalisation and altering the elimination/distribution mechanism. Notably, the clogP values for M6P, analogue 1 and 2 are -3.28, 0.10 and 3.29 respectively (Figure 1b). LogP is an estimate of a compound's overall lipophilicity, a value that influences its physiological properties such as solubility, permeability through biological membranes, hepatic clearance, and non-specific toxicity. Polar compounds with low logP have very low cellular permeability due to their low affinity for the lipid bilayers. Alternatively, lipophilic compounds with high logP have high affinity for the phospholipid phase facilitating their internalisation and prohibiting their escape into the aqueous basolateral side. Herein the lipophilic prodrug, analogue 2, will have improved cellular internalisation compared to its charged parent analogue, 1. Once internalised the bis(pivaloyloxyethyl) linkers of analogue 2 will be gradually prone to ester hydrolysis by microsomal esterases present within the intracellular compartments, resulting in the conversion to the charged parent analogue, analogue 1. Analogue 1 on the contrary would only target extracellular M6P/IGFII receptors, when administered directly, due to its low cellular permeability deemed to its low logP value.

Table 1 Ligand-Receptor Protein interaction energies obtained for M6P and each of the two analogues in domain 3 and domain 5 as determined from 100 ns of molecular dynamics simulation. Two ligands were placed into the dimer binding pocket, because the receptor is secreted as a dimer.

<table>
<thead>
<tr>
<th>Domain 3 Ligand-Receptor Protein interaction Energy (kcal/mol)</th>
<th>M6P</th>
<th>Analogue 1</th>
<th>Analogue 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ligand 1</td>
<td>-368.4</td>
<td>-309.3</td>
<td>-81.6</td>
</tr>
<tr>
<td>Ligand 2</td>
<td>-347.4</td>
<td>-304.3</td>
<td>-79.6</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Domain 5 Ligand-Receptor Protein interaction Energy (kcal/mol)</th>
<th>M6P</th>
<th>Analogue 1</th>
<th>Analogue 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ligand 1</td>
<td>-128.2</td>
<td>-44.1</td>
<td>-43.6</td>
</tr>
<tr>
<td>Ligand 2</td>
<td>-118.3</td>
<td>-74.0</td>
<td>-47.8</td>
</tr>
</tbody>
</table>

The extracellular region of the M6P/IGFII receptor is comprised of 15 repetitive domains and contains three distinct M6P binding sites located in domains 3, 5, and 9, with only domain 5 exhibiting preference for phosphodiesters. In order to assess our strategy to use the intracellular conversion of the produg analogue 2 to a high receptor binding phosphonate analogue 1, it is pivotal to examine the ligand-receptor interactions to validate the hypothesis that analogue 2 will have minimal interaction with the extracellular receptors. In the current study, we used six independent molecular dynamics simulations to study the ligand-receptor protein interactions of M6P, analogues 1 and 2 with domains 3 and 5 of the extracellular M6P/IGFII receptor (see Supporting Information for experimental details; section S9.1). Domain structures were adopted from previously reported studies and two ligands were placed into the dimer binding pocket, because the receptor is secreted as a dimer. Analogue 1 showed similar ligand-receptor protein interaction energies to M6P in domain 3 (Table 1). Importantly, the m-xylene ring of analogue 1 was positioned in the middle of the binding pocket further stabilising the binding of this compound in comparison to M6P (see Supporting Information; Figures S12 and S13). This is in accordance with the previous studies of other phosphonate analogues of M6P, which are reported to display higher affinity and stronger stability in human serum than M6P. The domain 5 binding pocket is larger than in domain 3, hence all the compounds displayed weaker interactions with the receptor and occupied more diverse positions in domain 5 due to the increased space (see Supporting Information; Figure S13). Furthermore, in the case of analogue 1 in domain 5, the simulations suggested that one of the two analogue 1 ligands (ligand 1) bound to the protein dimer has weaker interactions with the protein as it primarily interacts with the second molecule of analogue 1 (ligand 2). Overall, the simulations suggested that analogue 1 has high affinity towards domain 3 similar to M6P whilst the produg 2 has
weak interactions with both domains of the receptor (Table 1 and see Supporting Information; Figure S14). The molecular dynamics simulations further validated our aforementioned hypothesis that the prodrug will be internalised with minimal extracellular receptor-ligand interactions.

We next validated our hypothesis in a well-established in vitro model for wound healing using primary human dermal skin fibroblasts (HDF). In mammals, wound healing is not a regenerative process that restores normal tissue architecture, but a reparative process that results in scar formation.25 This process occurs in all tissues of the body in response to physical, chemical and biological stressors. Scar tissue is functionally and aesthetically inferior to normal tissue. It is a result of the excessive production of extracellular matrix (ECM) that occurs after injury.26 One of the most important proteins influencing the ECM architecture during wound healing is collagen I. Collagen I is synthesised predominantly by fibroblasts and its synthesis is largely regulated by cytokine transforming growth factor beta (TGFβ).27 TGFβ is secreted in an inactive form (LTGFβ), requiring enzymatic conversion to active TGFβ to effect a change in cell function. One of the methods of TGFβ activation involves binding of M6P residues within the N-linked oligosaccharides on latent TGFβ to the M6P/IGFII receptor.28 Since the M6P binding sites are involved in various steps of TGFβ activation and inactivation, it is believed that small molecule inhibitors that block the binding of M6P residues could present an opportunity to block the activity of TGFβ thereby reducing overproduction of an important profibrotic extracellular matrix protein collagen I. Cytotoxicity and cell viability of the analogues were initially assessed usingMTS and live/dead assays (see experimental details in Supporting Information; sections S2.1, S3.1 and S4.1). Previous studies characterising M6P binding affinity towards the M6P/IGFII receptor reported significant binding affinity at a concentration of 10 μM.9, 29, 30 This concentration was therefore selected for our in vitro studies. All compounds showed no effect on cell viability and proliferation both in the presence and absence of TGFβ (Figure 2 and see Supporting Information; Figure S15 respectively). Exposure to TGFβ in the absence of analogues 1 and 2 resulted in a reduction in HDF proliferation (see Supporting Information; Figure S4). This growth suppressive response has been previously reported in many cell types.31 The observed change in cell proliferation upon exposure to TGFβ influenced HDF cell morphology (and see Supporting Information; Figure S16b). Fibroblasts alter their morphology from stellate to dendritic upon exposure to various external cues caused by changes in actin polarisation and focal adhesion.32, 33 TGFβ has been shown to alter the morphology of many cell types including fibroblasts, potentially by inducing polymerisation of the actin cytoskeleton from globular to filamentous.34 Different factors such as cell motility and mechanical strain have also been reported to cause this alteration.34 In the present case, we observed a reversal of HDF cell morphology back to initial cell morphology without TGFβ stimulation when treated with the analogues 1 and 2 (Figure 3 for quantification of cell body area and see Supporting Information; Figure S16c-e for images). We next assessed if the observed change in morphology is correlated to collagen I gene expression using qRT-PCR, and if changes in collagen I gene expression could be altered by inhibition of TGFβ1 activity by targeting the M6P/IGFII receptor in the presence of the analogues (refer to Supporting Information for method; section S5.1). Indeed as previously reported, exposure of HDF to TGFβ (2 ng/mL) resulted in a significant increase in collagen I mRNA expression at 48 hours post-stimulation.27, 35 It is noteworthy that although collagen I gene expression was upregulated throughout the study period (72 hours), the optimal response was observed after 48 hours exposure to TGFβ (see Supporting Information; Figure S17). Therefore, the efficacy of the aforementioned compounds was assessed in the presence of TGFβ at 48 hours. TGFβ induced collagen I mRNA expression was downregulated significantly (p < 0.05) with the addition of prodrug analogue 2 (10 μM) with levels returning to that of normal untreated cells (Figure 4a). Downregulation was also observed for M6P however, the change did not reach statistcal significance (Figure 4a). This suggests that the variable responses that have been reported in the use of hydrolytically unstable M6P may be due to its relative instability and that the development of stable analogues may resolve this issue. Importantly, in the present case we observed no significant change in collagen I gene expression in HDF cells treated with analogue 1 (Figure 4a). This was expected given the low cellular permeability which is believed to affect the ligand-receptor protein interactions in cells. Next, we investigated if the observed change at transcription level would have a corresponding influence on protein translation. Changes in collagen I protein expression were quantified using immunoblotting (refer to Supporting Information for method; section S6.1). All protein expression studies were carried out at 72 h post-stimulation.
Significant upregulation in collagen I protein expression was observed post TGFβ1 stimulation (Figure 4b; column 2; p < 0.05) which is consistent with previous reports. Analogue 2 (10 µM) was observed to reduce TGFβ1 mediated upregulation of collagen I protein to non-stimulated levels (Figure 4b; column 5; p < 0.05). No significant changes were observed in the case of M6P or analogue 1. This further confirms that analogue 2 is a potent repressor of TGFβ1 induced collagen I synthesis and thus can ameliorate the profibrotic effects of TGFβ1 in human skin dermal fibroblasts.

In summary, we have developed a novel approach using an intracellular prodrug of M6P, analogue 2, to target M6P receptors. This approach overcomes the physiological problems associated with the hydrolysis of M6P whilst successfully targeting the receptors using an intracellular covalent of the analogue. We believe that this approach of intracellular drug covalence for receptor targeting will have far reaching implications in the design of highly potent drug candidates for enzyme replacement therapies of lysosomal storage diseases, to aid wound healing and in cancer therapy.

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Notes and references
The authors declare no competing financial interests.