



## A single injection of CM1021, a long half-life hepatocyte growth factor mimetic, increases liver mass in mice

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### ABSTRACT

Despite years of positive animal data, hepatocyte growth factor (HGF) has never been developed into a useful pharmaceutical, primarily due to its poor pharmacological properties. CM1021 is a fusion protein containing the K1 loop of HGF and the human IgG1 Fc region. The experiments described here demonstrate that CM1021 has the biological properties of HGF and the pharmacological properties of a monoclonal antibody. CM1021 stimulates scattering and branching morphogenesis in MDCK cells and stimulates liver growth in vivo. Unlike HGF, it is available via intraperitoneal injection and has an estimated half-life similar to an antibody.

Hepatocyte growth factor (HGF) plays a critical role in the development and maintenance of the lungs, liver, pancreas, heart and neural system [1]. Discovered in the late 1980s as a protein responsible for liver regeneration, the ensuing thirty years have seen a vast literature develop on the positive effects of HGF in a wide variety of diseases. In particular, multiple animal studies have demonstrated the protective and regenerative effect of HGF on the lung, suggesting that it may have utility in treating the persistent after effects of COVID19. HGF stimulates regeneration of the lung after lung reduction surgery in rats [2]. HGF stimulates proliferation of both alveolar type I and II cells [3]. HGF reverses fibrosis in bleomycin induced injury in the mouse [4]. Nonetheless HGF has never been developed into a useful therapeutic. Due to its complex structure, need for secondary activation and unfortunate binding to heparan sulfate proteoglycans, it has very poor pharmacological properties and persists in the blood for only a few minutes [5].

HGF is a complex molecule with a molecular weight of 80,000 [1]. It is synthesized as a precursor and is activated by several proteases into a two chain molecule with a disulfide link. The A chain contains four kringle structures. The B chain has similarity to a number of proteases, although it has no protease activity itself. The A chain is responsible for the high affinity of HGF for its receptor, c-met [6].

The A chain can be further subdivided into several regions. The N terminus (N) has a hairpin loop structure. The four kringle domains (1–4) have identical three dimensional structures but slightly different sequences. The N and K1 regions carry the high affinity binding site for the receptor. HGF binds to c-met as a dimer, thereby activating two

single copies of the receptor and initiating intracellular signaling [7,8].

HGF has a high affinity for heparan sulfate proteoglycans, expressed on the surface of most cell types [6]. This limits its diffusion and allows it to act in a very local manner, staying where it is synthesized. This property is important in embryogenesis where local structural and chemical gradients guide differentiation and morphogenesis. It unfortunately limits the usefulness of HGF when it is required to act at a distance or in higher concentrations.

The heparan sulfate binding sites of HGF are found in the N and K2 regions of the molecule. The K1 region, without the rest of the native HGF protein, does not bind to heparan sulfate yet binds and activates the c-met receptor [6]. This presents an opportunity to produce a molecule that has the appropriate pharmacological characteristics to make an HGF therapeutic.

IgG is one of the longest lived proteins in the blood, with a half-life of almost one month. This persistence results from its binding to the neonatal Fc receptor (FcRN), expressed in the early endosome. When proteins enter the cell, they are directed to the endosome for degradation. FcRN binds IgG and redirects it back to the cell surface, escaping degradation. Coupling a short lived molecule to the Fc region of IgG takes advantage of this system to increase the half-life of the shorter lived molecule [9,10].

CM1021 is a fusion between the K1 loop of HGF and the Fc region of immunoglobulin G (IgG). It is synthesized and secreted as a dimer. The experiments described here demonstrate that CM1021 has the biological properties of HGF and the pharmacological properties of an

*Abbreviations:* HGF, hepatocyte growth factor; FcRN, neonatal Fc receptor; TCEP, (tris(2-carboxyethyl)phosphine); IPF, Idiopathic pulmonary fibrosis.

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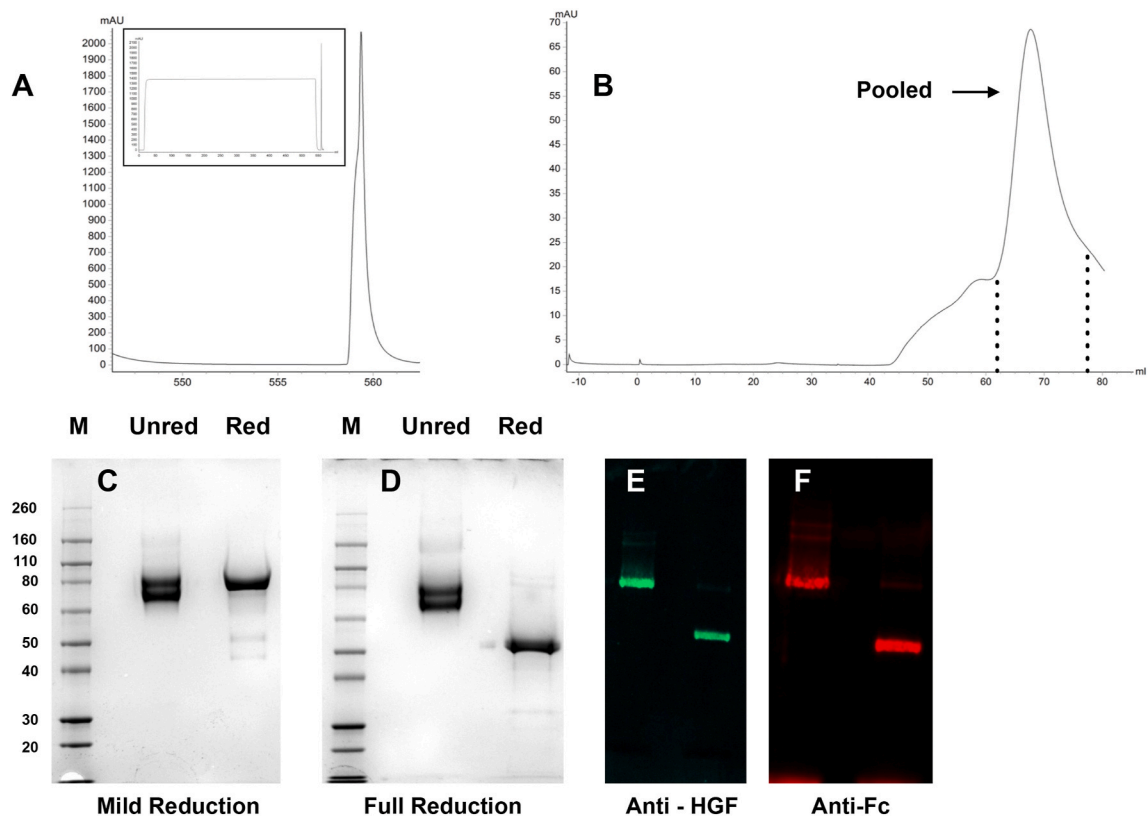
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**Fig. 1. Purification of CM1021.** A. Elution of CM1021 from protein A column. Inset shows the course of the entire binding and elution. B. Chromatography of the protein A eluate on Superdex 200. C. SDS PAGE of purified protein  $\pm 2.5$  mM TCEP. D. SDS PAGE of purified protein  $\pm 2.5$  mM TCEP plus  $80^\circ\text{C}$  for 10 min. E. Immunoblot of partially reduced and fully reduced CM1021 against an antibody to the K1 fragment of HGF. F. Immunoblot of partially and fully reduced CM1021 against an antibody to human IgG1 Fc.

immunoglobulin.

## 1. Materials and methods

**Materials** – Chemicals and rat tail collagen were obtained from SigmaAldrich. PureLink HiPure plasmid kit, Expi293 cells and transfection system, the Chromogenic Endotoxin Quant kit, Bolt bis-tris gels and cell culture medium were all obtained from ThermoFisher. HiTrap protein A FF columns, HiLoad Superdex 200 and the Akta pure system were obtained from Cytiva. All primary and secondary antibodies were obtained from Abcam. Biotin labelled c-met and FcRN were obtained from ACRO Biosystems. Human HGF was obtained from Peprotech.

**Plasmid synthesis** – The plasmid pK1Fc was synthesized by GenScript. It consists of amino acids 1–18 of the human albumin gene, amino acids 124 to 210 of human hepatocyte growth factor, a glycine serine rich linker of 50 amino acids, and amino acids 225 to 445 of the human immunoglobulin G1. The synthesized sequence was inserted into pcDNA3.3. Plasmid was amplified in *E. coli* and purified using PureLink HiPure kit (ThermoFisher).

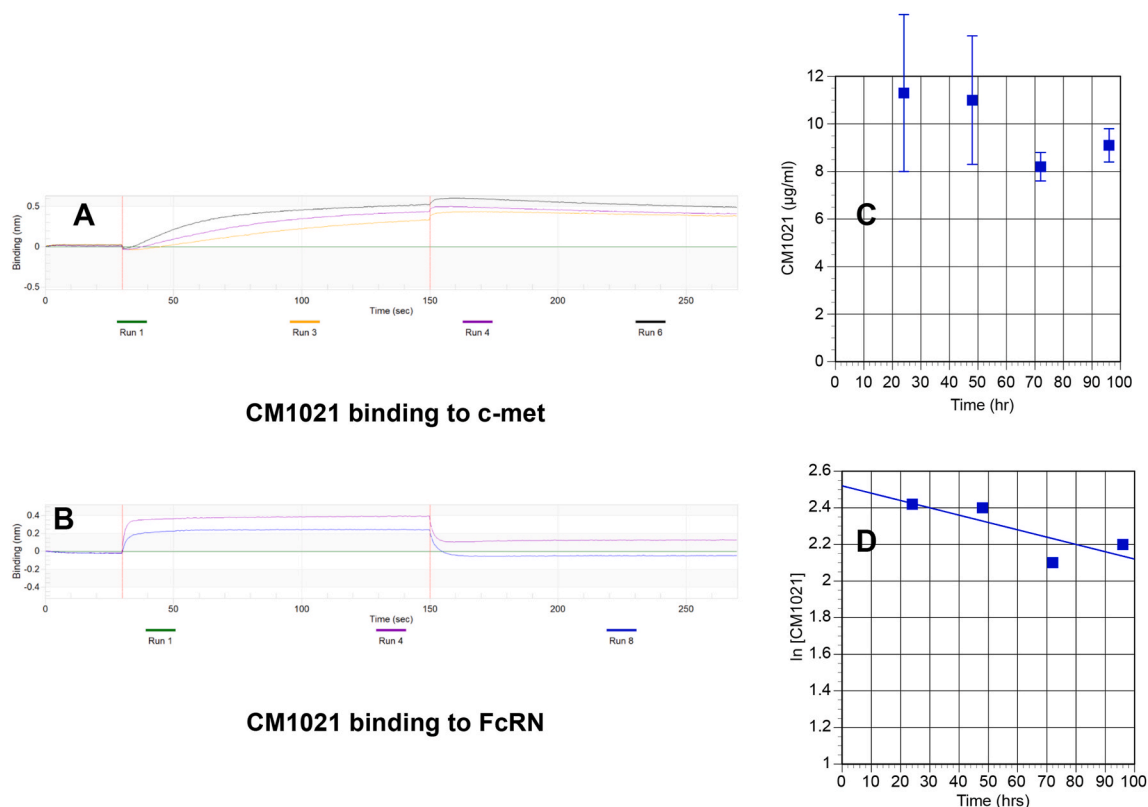
**Production of CM1021** – Expi293 cells were cultured and transfected using the complete Expi293 expression system according to the manufacturer's instructions (ThermoFisher). The transfected cells were cultured with shaking at  $37^\circ\text{C}$  for four days. The supernate, approximately 500 mls, was clarified by centrifugation and filtration before being applied to a 1 ml HiTrap Protein A FF (Cytiva) column using an AKTA Pure chromatography system (Cytiva). After application of the supernate, the column was washed with 20 column volumes of 20 mM HEPES, 150 mM NaCl, pH 7.4. The protein was eluted with 0.2 M glycine, pH 3.5. The fractions containing the protein were immediately neutralized by the addition of 0.3 vol of 1 M HEPES, pH 7.4. The fractions were pooled and applied to a calibrated HiLoad 16/600 Superdex

200 column. Fractions of the appropriate molecular weight were pooled and assayed with a SimpleStep human IgG ELISA (Abcam). The purified protein was subjected to two rounds of Triton X-114 to remove endotoxin [11]. Endotoxin was  $<1$  EU/ml after extraction as assayed with the Pierce Chromogenic Endotoxin Quant Kit (ThermoFisher). The solution was sterile filtered and diluted to 100  $\mu\text{g}/\text{ml}$ , frozen in liquid nitrogen and stored at  $-80^\circ\text{C}$ .

**Polyacrylamide gel electrophoresis and Western blotting** – Polyacrylamide gel electrophoresis was carried out using 4–12% Bolt bis/tris gels (ThermoFisher) and blotted using the iBlot Western system (ThermoFisher). Blots were probed with a goat anti-human IgG Fc antibody labelled with Dylight 650 (Abcam, ab98622) and with a rabbit monoclonal antibody to human HGF (Abcam, ab178395). The HGF blot used a donkey anti-rabbit secondary antibody labelled with Alexafluor 488. The gels and blots were imaged on an iBrightFL1000.

**Biolayer interferometry** – Biolayer interferometry was carried out using a Sartorius N1 system. Biotin labelled human c-met (Acro Biosystems) was bound to SAX high precision biosensors (Sartorius) at 10  $\mu\text{g}/\text{ml}$  in phosphate buffered saline, 0.01% Tween 20. Binding of CM1021 to c-met was also in PBS plus Tween. Biotin labelled human FcRN (Acro Biosystems) was bound to SAX high precision biosensors at 5  $\mu\text{g}/\text{ml}$  in the same buffer. Binding of CM1021 to FcRN was carried out in 25 mM NaAcetate, 25 mM  $\text{NaH}_2\text{PO}_4$ , pH 5.5, 150 mM NaCl, 0.01% Tween 20.

**Cell culture** – MDCK cells were obtained from the American Type Culture Collection and cultured in DMEM containing 5% FCS, pen/strep, 2 mM Glutamax (ThermoFisher). For scattering assays, cells were plated at low density and allowed to attach overnight. They were then washed three times with PBS and cultured in the absence of serum overnight. HGF (Peprotech) or CM1021 were added and the cells examined 24 h later.



**Fig. 2.** CM1021 binds to both c-met and FcRN. A. Biolayer interferometry (BLI) study demonstrating binding of CM1021 to probes loaded with biotinylated human c-met. B. BLI study demonstrating binding of CM1021 to probes loaded with biotinylated human FcRN. C. Measurement of CM1021 in mouse serum using an ELISA against human IgG. D. Replot of the data in panel C to estimate the half life of CM1021 in the mouse. The calculation from the slope of the line yields a  $t_{1/2}$  of 172hr.

For collagen gel culture, MDCK cells were trypsinized and diluted to 10,000 cells/ml in DMEM, PS, Glutamax and 0.1% FCS. Three ml of cell solution was diluted with three ml of 3% rat tail collagen solution (SigmaAldrich), neutralized with sterile 0.1 M NaOH. Twelve wells of a 24 well plate were filled with 0.5 ml of the solution. The gel was allowed to polymerize for 1 h at 37 °C. Control cultures (4 wells) were layered with DMEM, PS, Glutamax. HGF was added to the same solution at 125 ng/ml and added to 4 wells. CM1021 was added to the same solution at 125 ng/ml and added to 4 wells. Cultures were incubated at 37 °C in 5% CO<sub>2</sub> for seven days.

**Animal experiment** – The experiment was carried out in compliance with the NIH guidelines and was approved by the internal institutional animal care and use committee. Three male outbred mice were injected intraperitoneally with 4 mg/kg (115µg/0.4 ml) CM1021 dissolved in normal saline. Endotoxin was <1 EU/ml. Three male control mice were injected with saline alone. Approximately 200 µl of blood was drawn once per day for four days, allowed to clot for 1 h and centrifuged to recover the serum. Serum was frozen in liquid nitrogen until assay.

On day four, the mice were killed by cervical dislocation, weighed and their livers were carefully removed and weighed.

The concentration of CM1021 in the serum was determined using a SimpleStep ELISA against human IgG (Abcam). Control experiments carried out with purified CM1021 demonstrated that the human IgG ELISA accurately quantitated the test article. No reaction was obtained against the control mouse sera.

## 2. Results

**Production and purification of CM1021** – CM1021 was produced by transient transfection of pK1Fc into Expi293. Control experiments using GFP containing plasmids showed very high transfection efficiencies using this system. The cell suspension was harvested after 4 days and the

solution was clarified by centrifugation and filtration.

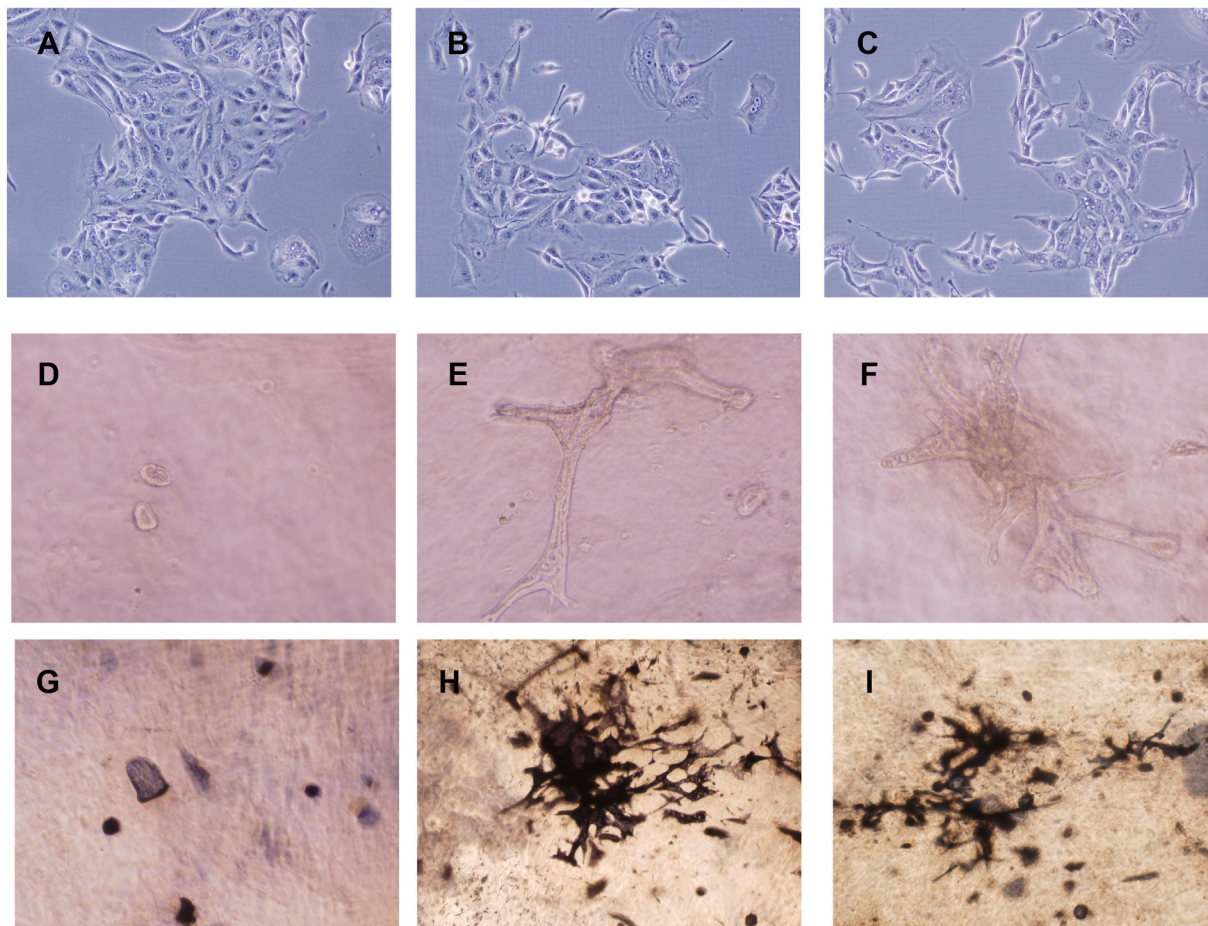
The clarified supernate was applied directly to a 1 ml protein A fast flow column at 2 ml/min. Bound protein was eluted in 1.5 ml (Fig. 1A) and was immediately neutralized. Eluted protein from approximately 1 L of culture fluid was applied to a Superdex 200 column (Fig. 1B) for further purification. Fractions containing protein of the appropriate molecular weight for the dimeric protein (77kD) were pooled. Eighteen milligrams of purified protein was recovered from 1 L of culture.

Fig. 1C shows an SDS polyacrylamide gel of unreduced protein and protein reduced with 2.5 mM TCEP, stained with Coomassie Blue. This mild reduction alters the double band to a single band. Fig. 1D shows an unreduced lane and one treated with 2.5 mM TCEP plus heating at 80 °C for 10 min. This is sufficient to fully reduce and dissociate the dimer into the monomer, as expected. This confirms that CM1021 is synthesized and secreted by the Expi293 cells as a dimer.

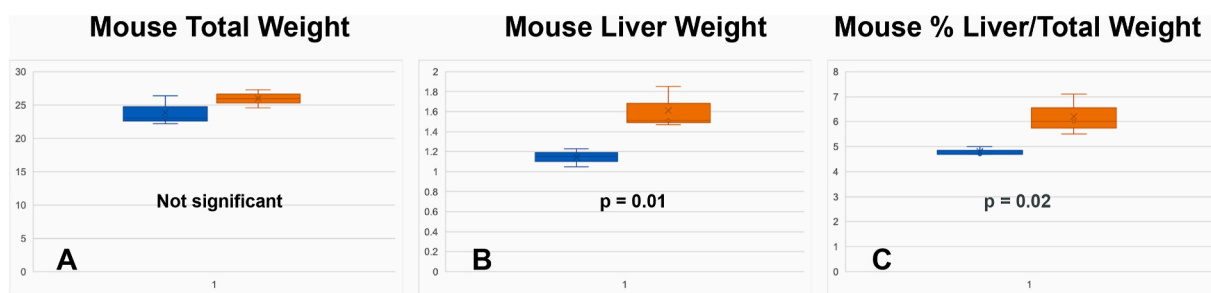
Subsequent experimentation determined that at least one, if not both, of the interchain disulfide links in the Fc portion of the molecule was reduced, accounting for the doublet in the absence of additional reduction and some polymerization occurred on storage (data not shown). Fig. 1E and F shows two immunoblots, the first lane in each treated with 2.5 mM TCEP and the second lane treated with 2.5 mM TCEP plus heating at 80 °C for 10 min. Fig. 1E was probed with an antibody to the K1 region of human HGF. Fig. 1F was probed with an antibody to human IgG Fc. The identical bands in both blots demonstrates the hybrid nature of CM1021.

An important objective of this project is for CM1021 to have properties of both HGF and an antibody so it must bind to both the HGF receptor, c-met, and to the neonatal Fc receptor, FcRN. A biolayer interferometry study examining binding to these receptors confirms this. Fig. 2, panel A, demonstrates binding of CM1021 to immobilized c-met. This experiment was intentionally set up to demonstrate binding of the dimer to c-met by loading the probe in a relatively high concentration of





**Fig. 3. CM1021 elicits scattering and branching morphogenesis in MDCK.** A – C. Scattering of MDCK in the absence (A) or presence of 125 ng/ml HGF (B) or 125 ng/ml CM1021. D – F. Branching morphogenesis of MDCK suspended in 1.5% collagen gels for seven days in the absence (D) or presence of 125 ng/ml HGF (E) or 250 ng/ml CM1021. 100× magnification. G – I. Cultures similar to those in D – F but stained with MTT for 2 h s. 40× magnification.



**Fig. 4. IP injection of CM1021 stimulates liver growth in mice.** Mice received a single injection of 4 mg/kg CM1021 at time zero. Mice and livers were weighed at 96 h. A. Control and treated mouse weights. Two samples are not statistically different. B. Control and treated mouse liver weights. Two samples are significant at p = 0.01. C. Control and treated mouse liver weight relative to total mouse weight. Two samples are significant at p = 0.02.

C-met. There are three phases to each binding test: the baseline, binding and dissociation. In the first phase, the probe is immersed in buffer to establish a baseline. In the second phase, the probe is immersed in the CM1021 solution. In the third phase, the probe is removed from the CM1021 solution and reimmersed in buffer, the ligand dissociates. Because CM1021 is binding as a dimer, the dissociation is very slow.

In Fig. 2, panel B, the binding of CM1021 to FcRN is demonstrated. In this case, Fc can also bind to two copies of the FcRN, so the probe was loaded with a lower concentration of FcRN [12]. Binding is carried out at pH 5.5, modeling the pH of the endosome. In the third phase, at pH 7.4, CM1021 readily dissociates from the receptor.

A simple in vivo demonstration of the effect of FcRN binding is

shown in Fig. 2C and D. Three mice were injected intraperitoneally with CM1021 and the concentration in the blood was measured over the next four days. Fig. 2C shows the raw data. There is little change in the concentration over this period. Fig. 2D shows a replot of the data in 2C to estimate the half-life of the CM1021. A calculation using the slope of the line in Fig. 4B estimates the half-life at 172 h. This is a crude estimate and the half-life is more properly determined from a longer experiment in mice expressing the human FcRN. Nonetheless, CM1021 clearly has a half-life that is several hundred times longer than native HGF. Not only is HGF unavailable via IP injection, it has a half-life of less than an hour in any organism.

There are three definitional experiments for HGF: scattering of cells

in standard cell culture [13], branching morphogenesis in collagen gels [14] and stimulation of cell division in the liver [5,15,16]. Fig. 3 A - C demonstrates scattering of MDCK cells by both HGF and CM1021. In the absence of HGF, MDCK cells grow as tight colonies and, if allowed to grow to confluence on a filter, develop a measurable electrical resistance across the monolayer. Stimulation of the c-met receptor, by either HGF (Fig. 3B) or CM1021 (Fig. 3C) elicits an internal signaling pathway that weakens the intercellular junctions and allows the cells to move away from one another.

Branching morphogenesis is a more complex exhibition of the scattering behavior described above. When MDCK cells are embedded in a collagen gel, they initiate a complex, spatially and temporally regulated signaling system to form branched tubules. Most organs depend on tubule formation at some point in their development: the lungs, the liver, the kidney. This is controlled by HGF. Fig. 3D demonstrates that in the absence of HGF signaling, MDCK cells in collagen form small cell discs, termed acini. In response to c-met signaling, initiated by either HGF (Fig. 3E) or CM1021 (Fig. 3F), over the course of a few days, the cells form large complex structures with clearly visible lumens. Fig. 3 G - H shows one week cultures stained with MTT to visualize the size and complexity of the structures.

HGF was originally defined in relation to its ability to initiate hepatocyte division [17]. Several studies have demonstrated that infusion of HGF can stimulate hepatocyte division *in vivo*. These are usually fairly complicated experiments due to the limitations of HGF. For example, Fujiwara, et al. used multiple intravenous injections [5,15], Roos, et al. used minipumps plus dextran sulfate to infuse over 72 h [5]. Patijn, et al., used the most complicated arrangement where multiple rats were cannulated for continuous infusion [16]. Fig. 4A-C shows that a single IP injection of CM1021 stimulates division in the liver and that the results are almost exactly comparable to those obtained by Roos, et al. with continuous infusion [5]. After 96 h s, livers in the treated mice were almost 40% larger, on average, than the untreated controls.

### 3. Discussion

HGF plays a central role in the development of the organism [1]. Knockouts are embryonically lethal and conditional knockouts have demonstrated an essential utility in organ generation and repair. Multiple laboratories have suggested the use of HGF as a therapeutic in lung [18], liver [1] and heart repair [19], among others, yet HGF has never been developed into a pharmaceutical. Its complex biology, binding to heparan sulfates, and subsequent short half-life have precluded its use and forced investigators to turn to more complex procedures such as continuous infusion or gene therapy.

To surmount these problems, a few laboratories are developing alternatives to HGF itself. For example, two groups are developing small molecules that facilitate binding of HGF to its receptor, c-met [20,21]. Another is developing agonist antibodies to c-met [22].

The work described here seeks to develop a molecule that has the biological properties of HGF but the pharmacological properties of an immunoglobulin. The production and purification are virtually identical to monoclonal antibody production. Protein A chromatography is the backbone of that industry. There is vast experience in process development and regulatory affairs available in the contract development and manufacturing organizations that can facilitate rapid development of CM1021. The binding study and the *in vivo* half-life estimation confirm that CM1021 takes advantage of FcRn, with a several hundred fold increase in the half-life. Most importantly, CM1021 closely mimics the major biological indicators of HGF action. Additional, more extensive studies will fill in the details of CM1021 action but these studies demonstrate that CM1021 fulfills the broad requirements that initiated its construction.

CM1021 may find utility in a number of fibrotic diseases that have been shown to respond to HGF but it may be particularly useful in pulmonary fibrosis; not only classical idiopathic pulmonary fibrosis

(IPF) but in the expected wave of lung fibrosis resulting from COVID19 [23-25]. IPF is an essentially incurable disease. The two FDA approved drugs for its treatment, nintedanib and pirfenidone, only slow progression but cannot reverse existing disease [26,27]. Interstitial lung disease is a common finding in patients with post acute sequelae of COVID19. HGF is traditionally seen as opposing TGF $\beta$  signaling [28]. TGF $\beta$  is one of the main perpetrators of fibrotic disease and not only stimulates the synthesis of matrix proteins, it also induces inhibitors of HGF activation, such as plasminogen activator inhibitor 1 [29]. A molecule that does not require activation, such as CM1021, may provide the excess of c-met signaling required to reverse fibrosis.

### 4. Conclusions

Despite years of work demonstrating the positive effects of HGF on cellular regeneration, there are still no useful pharmacological analogs of HGF. CM1021 demonstrates the biological properties of HGF coupled with a long, useful half-life *in vivo*. CM1021 may find use in a variety of intractable maladies, such as idiopathic pulmonary fibrosis.

### Ethics

The mouse experiment was carried out in compliance with the NIH guidelines and was approved by the internal institutional animal care and use committee.

### Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: James H. Kelly, PhD, is the founder and shareholder of Cell Machines, Inc.

### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bbrep.2021.101186>.

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