Reduction of liver fibrosis by rationally designed macromolecular telmisartan prodrugs

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At present there are no drugs for the treatment of chronic liver fibrosis that have been approved by the Food and Drug Administration of the United States. Telmisartan, a small-molecule antihypertensive drug, displays antifibrotic activity, but its clinical use is limited because it causes systemic hypotension. Here, we report the scalable and convergent synthesis of macromolecular telmisartan prodrugs optimized for preferential release in diseased liver tissue. We have optimized the release of active telmisartan in fibrotic liver to be depot-like (that is, a constant therapeutic concentration) through the molecular design of telmisartan brush-arm star polymers, and show that these lead to improved efficacy and to the avoidance of dose-limiting hypotension in both metabolically and chemically induced mouse models of hepatic fibrosis, as determined by histopathology, enzyme levels in the liver, intact-tissue protein markers, hepatocyte necrosis protection and gene-expression analyses. In rats and dogs, the prodrugs are retained long term in liver tissue, and have a well-tolerated safety profile. Our findings support the further development of telmisartan prodrugs that enable infrequent dosing in the treatment of liver fibrosis.

Liver cirrhosis, the final stage of hepatic fibrotic disease, is a rising global health issue that claimed over 1 million lives (2% of global deaths) in 2010-11. Within 5 years of being diagnosed with compensated cirrhosis, the overall patient survival rate is 45%. This disease, for which there are currently no Food and Drug Administration (FDA)-approved therapies, can result from several insults, including non-alcoholic steatohepatitis (NASH), alcoholic liver damage, viral hepatitis and toxic damage (Fig. 1a). The pathogenesis involves deregulated extracellular matrix production, including activated hepatic stellate cells (HSCs) and stromal fibroblasts. In preclinical studies, inhibition of components of the renin–angiotensin system (RAS) with small-molecule angiotensin II antagonists has been shown to limit stromal cell activation and proliferation, inflammation and oxidative stress associated with liver fibrosis12-14. For example, telmisartan (TEL), a known angiotensin II type 1 receptor blocker (ARB) and a widely used antihypertensive drug, has been reported to ameliorate hepatic fibrosis15. Despite this potential application, the clinical translation of ARBs, including TEL, for non-cardiovascular applications has been hindered by dose-limiting hypotension16-17. Such limits are even more problematic in advanced liver fibrosis where systemic hypotension often develops and is further accentuated by systemic drug accumulation due to decreased liver metabolism18.

To tackle these challenges, we set out to design a macromolecular TEL prodrug with an optimized biodistribution (BD) to fibrotic tissue and a tunable pharmacokinetic (PK) profile to ensure preferential release of active TEL in the diseased microenvironment. We envisioned that a macromolecular-conjugate-mediated ARB delivery strategy may overcome the poor therapeutic index often associated with therapies for chronic fibrotic disorders, and provide a clinically translatable therapeutic technology for liver fibrosis19.

Macromolecular drug carriers can alter the biodistribution of small-molecule drugs, with the liver being a major reservoir and clearance organ for neutrally charged ~10–50 nm objects20-21. Moreover, macromolecules in this size range are able to pass the leaky vasculature associated with chronic inflammatory disease and accumulate in the fibrotic microenvironment12-17. Despite these advantages, there is often an inverse relationship between scalability and the potential for modularity and complexity of macromolecular prodrugs that limits the rational and modular optimization of material properties in vivo and ultimately hinders their clinical translation (Fig. 1a). Materials that physically encapsulate their therapeutic payload have achieved clinical success in specific disease contexts; however, their formulation depends on the chemical properties of the drug, which limits the ability to vary properties such as drug identity, hydrodynamic diameter ($D_h$) and drug release kinetics (Fig. 1a, top left)10. In contrast, the reproducible scaling of advanced material architectures that rely on linear multi-step synthetic routes is difficult and may preclude rapid property optimization (Fig. 1a, bottom right)12. Several recent discussions have highlighted the fact that most nanomedicines reported in the literature do not translate to clinical applications10-20. Although this may be expected for experimental drugs, in our view, this dearth of examples is in part due to a lack of modular synthesis strategies for macromolecular prodrugs that are both versatile and scalable. Here, we present such a strategy for the rational design and optimization of a TEL-based convergent prodrug that reverses liver fibrosis in vivo. Our synthesis is demonstrated to be highly reproducible up to the 100 g scale, which establishes a benchmark for the scalable synthesis of complex macromolecular prodrugs required for drug development and in vivo safety pharmacology studies.
**Fig. 1** Rational design of TEL-x-MM and semi-batch synthesis of TEL-x-BASP. a, Diagram depicting the inverse relationship between scalability and potential for modularity and functional complexity in carriers for drug delivery. Convergent macromolecular prodrugs can potentially satisfy both demands. b, Synthesis of telmisartan macromonomers (TEL-MM) via esterification and CuAAC: (i) EDC·HCl, DMAP, CH2Cl2, 16 h, 73–93%; (ii) CuOAc, CH2Cl2, 1 h, 43–62%. Ester linkages between TEL and polymer increase in lability due to stronger electron-withdrawing groups (left to right). c, BASP synthesis via polymerization of TEL-x-MM (1) followed by semi-batch addition of AXL to induce crosslinking (2).

BASPs have chemically tunable release kinetics in vitro. Our approach involves a unique macromolecular architecture—the brush-arm star polymer (BASP)—the synthesis of which was intended to be modular and scalable\(^2\). BASPs are prepared in a convergent ring-opening metathesis polymerization (ROMP) process whereby norbornene-based drug-conjugated macromonomers (MMs) are polymerized to generate living bottlebrush polymer ‘arms’ that are subsequently crosslinked\(^3\). The composition and size of BASPs depends on the macromonomer and crosslinker structures and the ratio of polymerization initiator to macromonomer and crosslinker, respectively, all of which can be optimized in parallel for specific applications. Moreover, this approach enables rational control of drug release kinetics through linker design. For example, TEL contains a carboxylic acid functional group that we reasoned could serve as a linker site for conjugation via esterification (Fig. 1b). To identify the ester structure with optimal TEL release kinetics and therapeutic index in vivo, we began our study with the synthesis of three azide-functionalized (N\(_3\)) TEL-esters—TEL-1-N\(_3\), TEL-2-N\(_3\), and TEL-3-N\(_3\).
TEL-2-N and TEL-3-N —from alcohols 1, 2 and 3, respectively (Fig. 1b). These compounds were devised to provide increasing rates of esterase-mediated hydrolysis (that is, TEL release) via successive stabilization of the alkoxyide hydrolysis products through both resonance and inductive effects. To test the validity of this design, TEL-1-N, TEL-2-N and TEL-3-N were conjugated to alkyne-containing norbornene-polyethylene glycol (PEG) macromonomer 4 via copper-catalysed azide alkyne cycloaddition (CuAAC) to yield water-soluble macromonomers TEL-1-MM, TEL-2-MM and TEL-3-MM (Fig. 1b). The pseudo-first-order rate constants (±s.d.) for release of TEL from these macromonomers (in porcine liver esterase (100 units ml⁻¹) at 37 °C in phosphate-buffered saline (PBS) solution at pH 7.6) were 2.9±0.1×10⁻⁴ min⁻¹ (TEL-1-MM), 4.6±0.4×10⁻⁴ min⁻¹ (TEL-2-MM) and 8.8±0.7×10⁻⁴ min⁻¹ (TEL-3-MM) (Supplementary Fig. 1). Thus, the electron-deficient difluorophenyl-based linker (TEL-3-MM) provides a threefold greater release rate than the tetraethylene glycol linker (TEL-1-MM) under these conditions. Moreover, while all of these MMs showed nearly complete TEL release after ~1 day in the presence of esterase in vitro, they showed minimal release (<15%) after nearly a week in PBS buffer at 37 °C in the absence of esterase. Given that esterases are typically present at lower levels in plasma and at higher concentrations in intracellular lysosomal compartments of inflamed tissue, our strategy could favourably preclude the dose-limiting blood-pressure-lowering systemic effects of TEL by a preferential esterase-mediated release of TEL in the fibrotic liver microenvironment.

Next, we turned our attention to the synthesis of TEL-x-BASPs (where x corresponds to linker 1, 2 or 3) via ROMP of these TEL-ester macromonomers followed by crosslinking with a degradable acetal-based crosslinker (AXL) (Fig. 1c). With the ultimate goal of clinical translation of these materials, we developed an operationally simple and scalable semi-batch BASP synthesis protocol. In a typical reaction, Grubbs third-generation catalyst (G3, Fig. 1c) was added to a 0.05–0.1 M solution of TEL-MM (20 mg to 2 g) in 1,4-dioxane (G3:TEL-MM=1:N) to generate a living TEL-bottlebrush polymer with an average degree of polymerization of N. Next, a 0.1 M solution of AXL (m equiv.) in 1,4-dioxane was added by syringe pump at a rate of 1 equiv. min⁻¹ (Fig. 1b). After completion of the crosslinking process as determined by gel permeation chromatography (GPC) (Supplementary Fig. 2), ethyl vinyl ether (EVE, ~100 equiv.) was added to quench the reaction. We screened m and N values to identify suitable ratios for the synthesis of TEL-BASPs with narrow size distributions and D₅₀ of ~25–30 nm, as this size range is considered optimal for passage through the leaky vasculature of fibrotic diseased tissues. We were particularly interested in understanding the impact of m and N on BASP homogeneity, both in terms of size and size distribution, as well as bottlebrush-to-BASP percent conversion p. Using TEL-2-MM as a test macromonomer, TEL-2-BASPs were prepared using m=7, 10 and 13; for each m value a variety of N values were evaluated (Table 1). GPC was used to assess p (Supplementary Fig. 3), while dynamic light scattering (DLS) was used to measure D₅₀ as functions of m and N. For the range of m and N studied, p varied from 82% to 97% (Table 1). Larger p values were obtained with higher N and lower m. D₅₀ was fairly consistent, ranging from ~20 to 30 nm; however, for a given m, D₅₀ increased with N (Supplementary Figs. 8–16). Although it is possible to achieve p values as high as 97% in this system, this increased conversion comes at the expense of the TEL mass fraction, which generally decreases with increased N (Table 1). We conducted control experiments to confirm that the semi-batch protocol described herein is superior (as assessed by p value and dispersity) to our previous methods for BASP synthesis (Supplementary Fig. 4). Based on these results, we proceeded with the m=10, N=9 stoichiometry, as it provided an optimal combination of p (84% wt/wt) and TEL loading (11% wt/wt theoretical; 10.4±1.1% wt/wt experimental, see Supplementary Section H for details).

### Table 1: Effect of precursor stoichiometry on BASP properties

| m (TEL-2-MM:G3) | N (AXL:G3) | p (%) by weight | D₅₀ (nm) | Theoretical TEL (% by weight) |
|-----------------|------------|----------------|----------|-------------------------------|
| 7               | 7          | 82             | 22±3     | 11                            |
| 7               | 14         | 90             | 24±7     | 9.4                           |
| 7               | 21         | 95             | 27±10    | 8.4                           |
| 7               | 30         | 97             | 28±7     | 7.6                           |
| 10              | 9          | 84             | 24±5     | 11                            |
| 10              | 20         | 88             | 28±11    | 9.5                           |
| 10              | 30         | 91             | 28±11    | 8.5                           |
| 13              | 13         | 82             | 24±7     | 11                            |
| 13              | 24         | 87             | 27±8     | 9.6                           |
| 13              | 30         | 87             | 36±16    | 9.2                           |
| 13              | 40         | 90             | 36±18    | 8.5                           |

*Conversion from brush to BASP determined by GPC (DMF). *Hydrodynamic diameter determined by DLS in H₂O; error is reported as polydispersity from a regularization fitting method.

**TEL-2-BASP displays optimal liver BD and PK.** To select the optimal TEL linker for scale-up, the BD and PK profiles of TEL-2-BASP and TEL-3-BASP were investigated; TEL-1-BASP was not pursued further based on the sluggish TEL release kinetics observed for TEL-1-MM. Fluorophore-conjugated TEL-2-BASP and TEL-3-BASP were prepared from TEL-2-MM and TEL-3-MM, respectively (m=10), a cyanine7.5-fluorophore-labelled (Cy7.5) macromonomer (0.01 equiv., see Supplementary Section B for synthetic details) and AXL (N=9 equiv.) following our optimized semi-batch protocol described in the previous section. After dialysis, sterile filtration and lyophilization, TEL-2-BASP and TEL-3-BASP were examined in vivo. The lyophilized BASP product was reconstituted in PBS (40 mg ml⁻¹), sterile-filtered and administered intravenously (i.v., 200 mg kg⁻¹) to BALB/c mice (n=15) with blood and liver tissue samples collected for longitudinal liquid chromatography with tandem mass spectrometry (LC–MS/MS) measurements of both released and polymer-conjugated TEL (see Supplementary Section 1.2 for additional details). As shown in Fig. 2a, over the first 10 days following administration, TEL-3-BASP released significantly more TEL in blood compared to TEL-2-BASP. Given our goal of reducing the concentration of free TEL in blood, we selected TEL-2-BASP for analysis of liver PK (Fig. 2b). The results from this study consistently showed ~100-fold greater concentration of released TEL in the liver compared to blood samples. In addition, the concentration of polymer-conjugated TEL was >100-fold greater than released TEL in the liver, even 10 days post i.v. administration. The concentration of released TEL reached a steady state over this time period. These data suggest that TEL-2-BASP acts as TEL-releasing depot in the liver, which we hypothesized would enable infrequent dosing compared to generic TEL. Lastly, ex vivo, near-infrared fluorescence images (Cy7.5-fluorophore: λₐ= 700 nm, λₑ= 800 nm) of organs collected from a representative animal killed 7 days post TEL-2-BASP administration revealed preferential accumulation of fluorescence in the liver and the spleen (Fig. 2c).

**TEL-2-BASP synthesis is scalable.** Having identified TEL-2-BASP as our optimal candidate for further translation, we turned our attention to scaling up the synthesis of this macromolecular prodrug. We first investigated the performance of our optimized protocol in the context of a 2.0 g scale TEL-2-BASP synthesis. DLS and transmission electron microscopy (TEM) analysis of TEL-2-BASP prepared on the 0.02 g scale (Fig. 3a) versus the 2 g scale (Fig. 3b) revealed very similar particle sizes and size distributions; p was also similar for these batches. Next, the same procedure was repeated on
Here again, the TEL-2-BASP produced from this batch had the same size and size distribution as the smaller-scale batches (Fig. 3c); however, in this reaction $p$ was improved to 93%. We believe that the improved conversion in the larger-scale process is due to superior mixing achieved by the use of a shaft-driven mechanical stirrer rather than a magnetic stir bar. Altogether, these results clearly demonstrate the scalability of our brush-first ROMP TEL-2-BASP synthesis protocol.

TEL-2-BASP is well tolerated in mice. To determine the safety and efficacy of TEL-2-BASP, we conducted single and repeat-dose safety and toxicology studies in mice. First, to benchmark the potential blood-pressure-lowering effects of TEL-2-BASP versus generic TEL (administered at the equivalent dose to the human therapeutic dosage of 10 mg kg$^{-1}$ perorally), three groups of mice ($n=8$ per group) were dosed with either vehicle control, generic TEL, or TEL-2-BASP at three times the human therapeutic dosage equivalent to generic TEL (300 mg kg$^{-1}$ BASP based on 10% TEL loading). As expected, administration of generic TEL led to a statistically significant, transient drop in both systolic (Fig. 4a) and diastolic (Fig. 4b) blood pressures (two-tailed Student’s $t$-test, $P<0.0001$) 2 h after administration and returned to normal physiological levels within 24 h, consistent with a reported $t_{1/2}$ of 6 h and once-daily dosing. In normotensive patients, such systemic blood pressure lowering limits the more widespread usage of generic ARBs to treat fibrotic disorders. In addition, blood pressure lowering is prohibitive for use in patients with advanced liver fibrosis/cirrhosis that have developed systemic hypotension. In contrast to generic TEL, even at three times the concentration of

Fig. 2 | PK and BD data for TEL-x-BASPs in healthy mice. a, Longitudinal whole blood PK measurements of released and conjugated TEL following a single i.v. dose (200 mg kg$^{-1}$ BASP) of TEL-2-BASP and TEL-3-BASP ($n=3$ animals per time point). b, Comparison of blood and liver tissue PK of released and conjugated TEL following TEL-2-BASP administration (200 mg kg$^{-1}$ i.v.). c, Ex vivo fluorescence imaging (800 nm) of mouse organs collected 7 days post administration (300 mg kg$^{-1}$ i.v.) of Cy7.5-conjugated TEL-2-BASP: (i) liver, (ii) spleen, (iii) kidney, (iv) bladder, (v) heart, (vi) lung, (vii) gut, (viii) brain, (ix) skeletal muscle. Units of radiant efficiency: $(p\text{s}^{-1}\text{cm}^{-2}\text{sr}^{-1})/\text{W m}^{-2}$. All data reported as mean ± s.e.m.

Fig. 3 | Scalable synthesis of TEL-2-BASP. a–c, Photographs of reactions, dynamic light scattering histograms, and transmission electron micrographs (uranyl acetate staining) of 20 mg (a), 2 g (b) and 100 g (c) batches. Scale bars, 2 cm (reaction photographs); 200 nm (micrographs). See Supplementary Figs. 5–7 for GPC traces of TEL-2-BASP.
generic TEL, TEL-2-BASP did not produce a drop in systolic or diastolic blood pressures after 2h (first dose) or following a repeat dosing (second dose) given to the same animals 7 days after the first dose. These results agree with the blood PK data for TEL-2-BASP (Fig. 2), and support our hypothesis that a macromolecular TEL prodrug with appropriate linker structure can prevent the undesired blood-pressure-lowering effects of generic TEL concomitant with achieving significant TEL exposure in fibrotic liver tissue.

To further assess the safety of TEL-2-BASP, mice were given one or multiple doses of the Cy7.5-fluorescently labelled compound. After a single dose, 25 different tissues were collected from each mouse at day 1, day 3 or weeks 1, 3, 5, 7, 9 or 12 post dosing. Mice receiving three repeat doses weekly had their tissues collected at weeks 3, 7 or 12 post dosing. The emission of the Cy7.5 label in the various tissues was assessed by whole organ fluorescence; liver, spleen, kidney and spleen 24h after single dose (300 mg kg\(^{-1}\)) and 7 weeks after repeat dosing (300 mg kg\(^{-1}\) BASP once a week for 3 weeks) showing no acute or chronic microscopic lesions or PEG-induced vacuolation. Quantification of Cy7.5 emission in SDS–PAGE resolved liver tissue homogenates at different times points (24h to 12 weeks) following a single dose of TEL-2-BASP (300 mg kg\(^{-1}\) BASP, n=2–3 animals per time point), resolved for blinded analysis by an American College of Veterinary Pathologist, which included assessment of the presence or absence of cellular cytoplasmic vacuoles that have been previously associated with PEGylated test articles in other settings. No PEG-induced vacuoles were observed in any tissues examined (Fig. 4c)\(^{30,31}\).

TEL-2-BASP is well tolerated in higher species. Having established that TEL-2-BASP is well tolerated in mice, we next conducted dose escalation and safety toxicology studies in Sprague–Dawley rats, a species commonly used for drug safety/toxicity assessment as part of Investigational New Drug (IND)-enabling studies. Following M3(R2) regulatory guidance\(^32\), rats were first dosed up to the maximum feasible dose (MFD), which is 5 ml kg\(^{-1}\) for a slow-push bolus i.v. injection. This dose corresponds to 500 mg kg\(^{-1}\) TEL-2-BASP, equivalent to 30 mg kg\(^{-1}\) i.v. of Investigational New Drug (IND)-enabling studies. Following M3(R2) regulatory guidance\(^32\), rats were first dosed up to the maximum feasible dose (MFD), which is 5 ml kg\(^{-1}\) for a slow-push bolus i.v. injection. This dose corresponds to 500 mg kg\(^{-1}\) TEL-2-BASP, equivalent to 30 mg kg\(^{-1}\) TEL, or TEL-2-BASP (green, repeat i.v. dosing, 3× 300 mg kg\(^{-1}\) BASP equivalent to 30 mg kg\(^{-1}\) of telmisartan i.v.) highlighting the blood-pressure-sparing TEL-2-BASP formulation. b, Diastolic blood pressure measurements in mice following administration with generic telmisartan (red, single dose, 1x, 10 mg kg\(^{-1}\)) or TEL-2-BASP (green, repeat dosing, 3x = 300 mg kg\(^{-1}\) BASP equivalent to 30 mg kg\(^{-1}\) TEL) highlighting the blood-pressure-sparing TEL-2-BASP formulation. c, Representative H&E stained photomicrographs (from one of three total animals per group) of mouse liver, kidney and spleen 24h after single dose (300 mg kg\(^{-1}\) BASP = 30 mg kg\(^{-1}\) telmisartan) and 7 weeks after repeat dosing (300 mg kg\(^{-1}\) BASP once a week for 3 weeks) showing no acute or chronic microscopic lesions or PEG-induced vacuolation. d, Quantification of Cy7.5 emission in SDS–PAGE resolved liver tissue homogenates at different times points (24h to 12 weeks) following a single dose of TEL-2-BASP (300 mg kg\(^{-1}\) BASP, n=2–3 animals per time point). e, Anti-PEG ELISA assay of liver tissue homogenate at different time points (24h to 12 weeks) to evaluate PEG clearance. f, Dose-range-finding study in rats showing TEL-2-BASP dose proportionality between 50 mg kg\(^{-1}\) BASP and 500 mg kg\(^{-1}\) BASP with dose-proportional pharmacokinetics. All data are reported as mean±s.d.; statistical analyses performed with two-tailed Student’s t-test (****P<0.0001; NS, not significant).
TEL-2-BASP shows sustained release and reduces fibrosis.

The in vivo activity of TEL-2-BASP was first assessed in the stellate animal model (STAM) metabolic mouse model of NASH, where the liver disease is driven by a combination of diabetes and a high-calorie diet\(^\text{[11]}\). Specifically, NASH was induced in male mice (C57BL/6) by injection of a streptozotocin (STZ, 200\(\mu\)g) solution 2 days after birth, followed by feeding a high fat diet (HFD) starting at 4 weeks of age. The animals (n = 7 per group) were maintained on HFD for 9 weeks and administered two doses of TEL-2-BASP (300 mg kg\(^{-1}\) i.v.) at the beginning of weeks 6 and 7 of the HFD. Animals were then killed and assessed for liver fibrosis by Picrosirius Red (PSR) staining (Supplementary Fig. 23a). Although the TEL-2-BASP treatment showed a statistically significant (P < 0.05) reduction of fibrosis compared to that of the untreated animals (Supplementary Fig. 23b), the STAM model displayed only a mild fibrosis phenotype, consistent with this being a diet-induced model of non-alcoholic fatty liver disease. Hence, we next turned our attention to the effects of TEL-2-BASP in a chemically induced carbon tetrachloride (CCL\(_4\)) mouse model, which is characterized by a pronounced fibrosis phenotype as evident from our biomarker analyses (vide infra, Fig. 5). Specifically, three groups of mice (n = 10 to 12 per group) were challenged with 1 ml kg\(^{-1}\) of CCl\(_4\) (i.p. twice weekly for 6 weeks) to induce liver fibrosis. Fibrosis was confirmed by week 4 (Fig. 6 and Supplementary Figs. 24–31) based on histology (H&E and PSR), immunohistochemistry and digital immunofluorescent quantitative analyses (\(\alpha\)-smooth muscle actin, nestin and collagen-1). During the last 2 weeks of continuous CCl\(_4\) administration, diseased animals were treated with equivalent amounts of TEL over a 2-week period: either 14 doses of generic TEL (10 mg kg\(^{-1}\) daily perorally, Fig. 5a, grey arrows) or two total doses of TEL-2-BASP (700 mg kg\(^{-1}\) i.v; 10% TEL loading wt/wt, Fig. 5a, orange arrows) leveraging the sustained release of TEL from TEL-2-BASP observed in Fig. 2b. It should be noted that a 700 mg kg\(^{-1}\) TEL-2-BASP dosage is feasible for human translation based on allometric scaling\(^\text{[12]}\). With our dosing regimen, all animals received, on average, the same amount of TEL over the 2-week period (140 mg kg\(^{-1}\)). In the CCI\(_4\) liver fibrosis model, TEL-2-BASP showed efficacy even during ongoing CCI\(_4\) intoxication based on blood biochemistry (Supplementary Fig. 32), liver function, histology and biomarker endpoints (Fig. 6 and Supplementary Figs. 24–31). Moreover, both generic TEL and TEL-2-BASP-treated groups showed no significant body weight change compared to healthy or untreated diseased animals over the course of the 14-day treatment regimen (Fig. 5a). Terminal liver tissue PK analysis of released and bound TEL revealed that more than 10% of the systemically injected dose of TEL-2-BASP was present in the diseased liver 7 days post last dosing (Fig. 5b). Moreover, the concentration of polymer-conjugated TEL (221,689 ng \(\text{g}^{-1}\)) was ~170-fold higher than the concentration of generic TEL (6,720 ± 619 ng \(\text{g}^{-1}\)) and the amount of released drug from TEL-2-BASP (1,843 ± 118 ng \(\text{g}^{-1}\)) was within threefold of generic TEL; the large concentration of TEL prodrug in the diseased liver acts as a depot to provide a constant therapeutic concentration of released TEL long after the generic TEL dosage is cleared.

Importantly, consistent with the superior biodistribution to diseased liver tissue, TEL-2-BASP exhibited similar to improved efficacy compared to generic TEL for all endpoints examined. At the 6-week endpoint, liver enzyme levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and total bilirubin (TB) were significantly decreased in TEL-2-BASP-treated animals compared to untreated or those treated with generic TEL (Supplementary Fig. 32). Analyses of intact-tissue protein biomarkers based on immunohistochemistry and quantitative digital imaging of immunofluorescence, as well as histopathology, confirmed that TEL-2-BASP exerted a significantly superior protective effect against periporal hepatocyte necrosis (Fig. 6 and Supplementary Figs. 24–31). It also was more efficacious in restoring glycogen content (Supplementary Fig. 33) compared to generic TEL. Histopathology scoring for necrosis and inflammation (Supplementary Figs. 27–29 and 34) and independent apoptosis scoring through cleaved caspase-3 quantification (Supplementary Fig. 30a) both showed more than a fourfold reduction in the liver cell death score for TEL-2-BASP-treated animals compared to tissue from the untreated groups. Moreover, stage-specific embryonic antigen 1 (SSEA1) levels were significantly decreased for TEL-2-BASP-treated animals compared to those treated with generic TEL (Supplementary Fig. 30b). Along with significant hepatocyte protection by TEL-2-BASP treatment, images stained for \(\alpha\)-smooth muscle actin, nestin and collagen-1 (Supplementary Fig. 31), as well as scoring of PSR histological images by a blinded pathologist (Fig. 6 and Supplementary Figs. 24–29), strongly support the notion that TEL-2-BASP efficacy is due at least in part to reversal of fibrosis. In a repeat CCI\(_4\) efficacy study, we found that TEL-2-BASP reduced mRNA expression from 13 genes representative of inflammation and extracellular matrix synthesis and turnover in fibrotic tissues, lending further credence to an anti-fibrotic mechanism of action via microenvironment modulation (Fig. 5c). From these data, collectively, we conclude that despite an infrequent dosing paradigm (weekly), the efficacy of TEL-2-BASP significantly exceeds that of generic TEL administered daily in this mouse model of liver fibrosis.

Outlook. We have shown that optimization of the design and synthesis of macromolecular telmisartan produgs can enable superior biodistribution and release of the active drug in fibrotic diseased tissues, concomitant with a low release of the active drug systemically. The superior efficacy and unique pharmacokinetic profile of TEL-2-BASP, with a depot-like release and long half-life of active released TEL in the diseased tissue, suggests that an attractive infrequent dosing schedule might be feasible in the clinic. Overall, TEL-BASPs may enable tissue-microenvironment-targeted therapies for chronic inflammatory and fibrotic conditions. Moreover, the modularity and scalability of BASP synthesis demonstrated here make BASPs attractive for a range of additional applications in prodrug design.
**Fig. 5** | Design and efficacy of TEL-2-BASP and TEL in a chemically induced CCl₄ mouse model. 

**a.** Experimental study design for the CCl₄-induced mouse liver fibrosis model showing body weight measurement for all treatment groups (healthy, untreated and test agents: TEL (10 mg kg⁻¹, peroral (p.o.) once per day (q.d.); total of 14 doses) and TEL-2-BASP (700 mg kg⁻¹ i.v. once per week (q.w.); total of two doses) during 6 weeks of CCl₄ intoxication; data reported as mean ± s.e.m. **b.** Terminal liver PK measurements (week 6) for TEL and TEL-2-BASP (released and conjugated TEL); data reported as mean ± s.e.m. **c.** Gene expression analysis from a repeat CCl₄ efficacy study comparing two doses of TEL-2-BASP (300 mg kg⁻¹ i.v. q.w.) with daily administration of TEL (10 mg kg⁻¹ p.o. q.d.; total of 14 doses). Relative expression levels of 13 genes that become over-expressed in fibrotic tissue and are suppressed by TEL-2-BASP are shown; data reported as mean ± s.d., statistical analyses performed with two-tailed Student’s t-test relative to levels of CCl₄ group (*P < 0.05, **P < 0.01, ***P < 0.001). Individual P values are noted above each bar when statistical differences are present.

**Methods**

Detailed methods are described in the Supplementary Information.

**General procedure for synthesis of TEL-MM.** A 20 ml scintillation vial was charged with one of three TEL-N₃ compounds (TEL-1-N₃, TEL-2-N₃ or TEL-3-N₃) or Cy7.5-N₃, PEG-Alk–MM and a stir bar. In a nitrogen-filled glovebox, the reagents were dissolved in CH₂Cl₂ (1 ml CH₂Cl₂ per 100 mg PEG-alk–MM), to which was added ~3 equiv. of copper(i) acetate. The reaction was stirred until consumption of PEG-alk–MM (~1 h) was observed by LC–MS analysis. As the relative extinction coefficients of the TEL-N₃ and PEG-alk–MM are vastly different (the former being much larger), it is important that a small amount of TEL-N₃ is visible by LC–MS. It is difficult to remove unreacted PEG-Alk–MM and its presence is detrimental to the subsequent ROMP processes. The crude product was purified by preparative-high-performance liquid chromatography (prep-HPLC) or prep-GPC. For material purified by prep-HPLC, fractions containing product were concentrated by rotary evaporation and the resulting residue was redissolved in CH₂Cl₂ and dried over sodium sulfate. After chromatography (prep-HPLC) or prep-GPC, the crude reaction mixture was first concentrated and dissolved in minimal CH₂Cl₂. The solution was passed through a short column of neutral alumina (~10% MeOH/CH₂Cl₂) and the eluent was concentrated by rotary evaporation. Then, after purification by prep-GPC, fractions containing product were concentrated by rotary evaporation. The resulting solid was washed with cold diethyl ether by centrifugation of solids and decanting of the ether. This wash step was repeated three times to afford the desired TEL-MM or Cy7.5-MM as white and green solids, respectively. For material purified by prep-HPLC, the reaction mixture was passed through a short column of neutral alumina (~10% MeOH/CH₂Cl₂) and the eluent was concentrated by rotary evaporation. Then, after purification by prep-GPC, fractions containing product were concentrated by rotary evaporation. The resulting solid was washed with cold diethyl ether three times (as above) to afford TEL-MM as a white powder.

**General procedure for synthesis of TEL-BASP.** TEL-2-MM (2.00 g, 9.9 equiv.) and Cy7.5-MM (20.1 mg, 0.1 equiv.) were added to a 40 ml vial charged with a Teflon-coated stir bar and septon. In a nitrogen-filled glovebox, the following solutions were added:...
On completion of AXL addition, the crosslinking reaction was allowed to proceed was diluted with nanopure H2O (~9.5 ml, 1:1 dilution) before being transferred to dialysis tubing (RC, 8 kDa molecular weight cutoff) for dialysis against TMB substrate was added and further incubated at room temperature. Stop Anti-PEG ELISA was performed on the liver homogenates following standard reagent, ThermoFisher Scientific) and normalized for protein concentration. homogenized and lysates prepared in T-PER reagent (tissue protein extraction (Fig. 4e and Supplementary Fig. 17). For the ELISA assay, liver tissues were BD and PK analysis in vivo. In vitro TEL release assay. Approximately 5 mg of a given MM (TEL-1-MM, TEL-2-MM or TEL-3-MM) was weighed into a clean 4 ml vial. Separately, a stock solution of porcine liver esterase (17 units per mg solid) was made from 24 mg of solid esterase powder and 4 ml of PBS (pH7.6) such that the final activity of esterase in solution was ~100 units/ml. Each vial containing 5 mg of a given MM was dissolved in one volume of the esterase stock solution. The polymer solutions were briefly vortexed and immediately portioned into 1 ml vials with Teflon-coated caps. All vials were sealed and placed in a 37 °C static incubation oven. At each time point, a vial for a given MM was removed from the incubation oven and cooled to room temperature. To each vial was added 200 μl dimethylsulfoxide, and the resulting solution was briefly vortexed before filtering through a 0.45 μm nylon syringe filter. Analysis by LC provided insight into the amount of TEL-MM remaining at a given time point.

BD and PK analysis in vivo. The lophylophorin BASP product was reconstituted in PBS (40 mg ml⁻¹), sterile-filtered, and administered i.v. (200 mg kg⁻¹) to BALB/c mice (n = 15) with blood and liver tissue samples collected for longitudinal LC–MS/MS measurements of both free (unconjugated) and polymers-conjugated TEL. The presence of polymer was quantified in liver homogenate by Cy7:5 imaging after resolution on an SDS–PAGE gel following a higher dose of TEL-2- BASP (300 mg kg⁻¹). Similarly, PEG clearance was measured by anti-PEG ELISA (Fig. 4c and Supplementary Fig. 17). For the ELISA assay, liver tissues were homogenized and lysates prepared in T-PER reagent (tissue protein extraction reagent, ThermoFisher Scientific) and normalized for protein concentration. Anti-PEG ELISA was performed on the liver homogenates following standard procedures (PEGylated protein ELISA kit, ab133065, Abcam). Briefly, test samples were added to a 96-well plate coated with monoclonal antibody specific to PEGylated protein. This procedure was followed by addition of biotinylated PEG-protein and incubation at room temperature for 30 min. The plates were washed to remove excess unbound protein and a solution of streptavidin–HRP conjugate was added. Following incubation, excess reagents was washed and TMB substrate was added and further incubated at room temperature. Stop solution was then added and optical density (OD) absorbance was measured at 450 nm using a SpectraMax plate reader.

CCL mouse model. Female mice (BALB/c) were ordered from Beijing Vital River Laboratory Animal Co. Ltd and housed at PharmaLegacy Laboratories in a specific room (required due to the toxicity of CCL). The animals were specific-pathogen-free and – 6 to 7 weeks old on arrival at the PharmaLegacy Laboratories vivarium, where they were housed in clear polycarbonate plastic cages (260 mm x 160 mm x 127 mm), with five animals per cage. All procedures were performed in accordance with PharmaLegacy regulations and were approved by PharmaLegacy Laboratories IACUC. A health inspection was performed on each animal, including evaluation of coat, extremities and orifices. Each animal was also examined for any abnormal signs in posture or movement. The period of acclimatization was 7 days. The rodent material was autoclaved corn cob bedding (Shanghai MaoSheng Biologic Science & Technology Development Co., Ltd) and was changed twice per week. The room was supplied with HEPA filtered air at a rate of 15–25 air changes per hour. The temperature was maintained at 20–26 °C (68–79 °F) with a relative humidity of 40–70%, the relative humidity being continuously monitored and recorded. Illumination was fluorescent light for 12 h light (8:00–20:00) and 12 h dark (cat. no. MO1-F). Animals were assigned to treatment groups (n = 10–12 animals per group) by randomization in BioBook software to achieve a similar group mean weight, which provided for control of bias. Additionally, disease animals (CCL) were assigned according to their ALT/AST levels first and body weight second after 4 weeks of CCL treatment. Animals were injected intraperitoneally with CCL, (i.p.) 1 ml kg⁻¹ (4 ml kg⁻¹ of 25% CCL in olive oil), twice per week for a total period of 6 weeks with test agents administered during the last 2 weeks of study. Induction of fibrosis was assessed first in both male and female BALB/c mice by PSR staining of harvested liver tissue. The aggressive, bi-weekly, intraperitoneal CCL, dosing regimen led to a more consistent level of fibrosis in female mice compared to male mice. The male mice displayed higher variability and mortality rates during the 6-week continued CCL induction. Hence, female mice were chosen so there would be consistency across treatment groups when comparing effect sizes. TEL was formulated from dry powder in 0.5% hydroxyl propyl methyl cellulose (HPMC) with 0.2% Tween-80 solution, and the test agent was administrated by daily oral gavage. TEL-2-BASP (lyophilized material) was reconstituted in PBS on the day of dosing (70 mg ml⁻¹ solution), sterile-filtered (0.22 μm) and dosed intravenously according to body weight (that is, 200 μl per 20 g animal; total of two doses). At the conclusion of the study, the whole liver tissue was flushed quickly with ice-cold PBS, blotted briefly on a paper towel, and weighed. Liver tissue was dissected into pieces: the right lobe was fixed in 10% neutral formalin; the left lobe was shock frozen in liquid nitrogen for tissue PK analysis. For tissue fixation, tissues were placed into 10% neutral buffered formalin (NBF; 20 mL volume) and incubated overnight (at least 12 h) at 4 °C. The following day, the tissue was moved to room temperature for 4–5 h, before being washed twice with PBS and stored at 4 °C for subsequent paraffin embedding and immunohistochemical processing. Animal blood samples (non-fasting) were collected at sacrifice for terminal PK analysis (whole blood in EDTA tubes) and plasma was processed by refrigerated centrifugation within 30 min of collection (2,000 g for 10 min at 4 °C). Plasma levels of ALT, AST and TB were measured using an automatic biochemistry analyser (HITACHI 7020).

Reporting summary. Further information on experimental design is available in the Nature Research Reporting Summary linked to this article.

Data availability. All data supporting the findings of this study are available in the paper and its Supplementary Information.

Received: 14 February 2018; Accepted: 19 July 2018; Published online: 20 August 2018

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**Acknowledgements**

Funding was provided by XTuit Pharmaceuticals. J.A.J acknowledges the National Institutes of Health (1R01CA220468-01) for support of this work. M.R.G. acknowledges the National Institutes of Health for a postdoctoral fellowship (1F32EB023101). H.V.-T.N thanks the National Science Foundation for a Graduate Research Fellowship. The authors thank R. Bronson for assistance with histopathology analysis.

**Author contributions**

M.R.G., J.L and J.A.J designed synthetic experiments. M.R.G., J.L., E.V., H.V.-T.N. and D.C.E synthesized materials. J.L., J.K.S.-S., P.W.K. and D.C.E. developed the scaled process and produced materials for the safety and toxicology studies. J.N.A. and P.B.-J. planned in vivo experiments. P.B.-J. and M.V.S. planned biomarker analyses. M.V.S., S.J.H., B.V., A.M.N., J.C.A. and J.B. performed biomarker analyses. All authors helped to analyse data. M.R.G., J.N.A., P.B.-J. and J.A.J wrote the manuscript. All authors read and edited the manuscript.

**Competing interests**

J.L., J.N.A., M.V.S., S.J.H., B.V., K.D.E., A.M.N., J.C.A., J.B., S.P., S.W.B., E.J.H., J.K.S.-S., P.W.K., D.E.C. and P.B.-J. are former employees and shareholders of XTuit Pharmaceuticals. P.B.-J. is President and Founder of Acrivon Therapeutics, J.A.J is a Co-Founder of Acrivon Therapeutics.

**Additional information**

Supplementary information is available for this paper at https://doi.org/10.1038/s41551-018-0279-x.

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1. Sample size
   Describe how sample size was determined.

   For the initial studies, we used n = 15 for mouse safety (BD/PK), n = 3 animals for 5 different time points (minimum number of animals used to obtain statistically significant PK and BD data). For rat safety and PK, n = 12 (6 animals per group per sex) were used for increased statistical significance when assessing safety of TEL-2-BASP in rodents. For dog safety, n = 2 (1 animal per sex) for dose-escalation studies and n = 4 (2 animals per sex) for repeat dosing. The minimum number of Beagle dogs were used while still obtaining statistically relevant data on the effects of repeat dosings at high TEL-2-BASP levels. For efficacy studies in mice, n = 7 male mice per group were used for the STAM NASH model and n = 10 - 12 animals per group were used for the CCl4 model to ensure that statistically significant results were obtained in the context of advanced disease progression.

2. Data exclusions
   Describe any data exclusions.

   Pathological scoring and the corresponding stained images (H&E and PSR) were analyzed for 6 (of the 10 - 12 total) random animals per group in the CCl4 study.

3. Replication
   Describe the measures taken to verify the reproducibility of the experimental findings.

   All data (blood/organ PK values, BD, body weights, blood pressure, gene expression, serum biochemistry and histology) are reported from a statistically significant group of animals (n = 3+) and are representative of results from different animals. Furthermore, PK profiles were consistent among a statistically significant number of grouped rodents (i.e. separate studies using healthy BALB/c mice and Sprague Dawley rats). Liver-fibrosis efficacy data, as judged by blood biochemistry and histological stained images, was consistent among two well-established models used to assess liver fibrosis (i.e. carbon tetrachloride and high-fat diet STAM models).

4. Randomization
   Describe how samples/organisms/participants were allocated into experimental groups.

   Animals were allocated into study groups on the basis of body-weight randomization. For the carbon tetrachloride efficacy study, diseased animals were randomized into groups first on the basis of ALT/AST levels, followed by secondary randomization by body weight.

5. Blinding
   Describe whether the investigators were blinded to group allocation during data collection and/or analysis.

   Blinding was not possible during dosing, as different concentrations of TEL-2-BASP needed to be used for various groups. However, blinding was used during the preparation of all histological scoring reports by Dr. Roderick Bronson (Board Certified Veterinary Pathologist).

Note: all in vivo studies must report how sample size was determined and whether blinding and randomization were used.
6. Statistical parameters

For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

- n/a Confirmed
  - The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)
  - A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
  - A statement indicating how many times each experiment was replicated
  - The statistical test(s) used and whether they are one- or two-sided
    - Only common tests should be described solely by name; describe more complex techniques in the Methods section.
  - A description of any assumptions or corrections, such as an adjustment for multiple comparisons
  - Test values indicating whether an effect is present
    - Provide confidence intervals or give results of significance tests (e.g. P values) as exact values whenever appropriate and with effect sizes noted.
  - A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)
  - Clearly defined error bars in all relevant figure captions (with explicit mention of central tendency and variation)

See the web collection on statistics for biologists for further resources and guidance.

7. Software

Policy information about availability of computer code

Describe the software used to analyze the data in this study.

- GraphPad Prism 7 software was used for the statistical analyses presented. NMR data was processed with MestReNova NMR software.

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). Nature Methods guidance for providing algorithms and software for publication provides further information on this topic.

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a third party.

Complete synthetic details for the brush-arm star polymers used in this study (along with relevant chromatographic, spectroscopic, and spectrometric characterization data), and photographs of the reaction setup on various scales, are provided.

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

- A monoclonal antibody specific to PEGylated protein was used for anti-PEG ELISA. 96-well plates pre-coated with this antibody were used as received from the vendor (Abcam, ab133065). In addition, standard antibodies were used directly from the vendors for the immunohistochemistry assays: cleaved caspase-3 (Cell Signaling Cat. #9661) and SSEA1 (Biolegend Cat. #12560).

10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used.

- No eukaryotic cell lines were used.

b. Describe the method of cell line authentication used.

- No eukaryotic cell lines were used.

c. Report whether the cell lines were tested for mycoplasma contamination.

- No eukaryotic cell lines were used.

d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by iCLAC, provide a scientific rationale for their use.

- No commonly misidentified cell lines were used.
### Animals and human research participants

Policy information about studies involving animals; when reporting animal research, follow the ARRIVE guidelines.

#### 11. Description of research animals

Provide all relevant details on animals and/or animal-derived materials used in the study.

For BD/PK and safety studies in mice, female BALB/c mice were used. For safety and toxicology studies in rats, male and female Crl:CD(SD) Sprague Dawley rats were used (ca. 9 weeks old upon initiation of the study). For safety and toxicology in dogs, male and female Beagle dogs were used (ca. 9 - 12 months old upon initiation of the study).

For efficacy in the STAM NASH mouse model, female C57BL/6 mice (14-day pregnant) were used, which afforded newborn male and female C57BL/6 for the study. For efficacy in the carbon tetrachloride (CCl4) mouse model, female BALB/c mice (7 - 8 weeks old upon initiation of the study) were used.

#### 12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants.

This study did not use human research participants.