Expanding small-molecule target space to mRNA translation regulation

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Multiple layers of regulation are in place on mRNA translation to ensure that cells respond in a fast manner to environmental cues in a tissue-specific and mRNA-selective manner. Here, we discuss mRNA translation regulatory mechanisms and potential drug-intervention selective targets. Taking on a new scientific rational of translation regulation and consequently a new target space, we have developed a unique discovery platform that is able to identify selective small molecule drugs that affect translation of specific proteins. This approach has enabled targeting of proteins that have been considered undruggable. Our discovery platform was repeatedly utilized to identify compounds in multiple therapeutic programs, including fibrosis, oncology, anti-virals and Huntington’s disease. In fibrosis, the lead compound ANI-21 has demonstrated a tissue-specific effect in lowering the translation of Collagen-I and superior efficacy over best standard of care, in both cell and animal models, mediated by a novel mechanism of action. This program is expected to enter clinical studies within 12–18 months.

Introduction

Traditionally, drug discovery programs focused mainly on targeting cell surface receptors or signal transduction proteins regulating gene transcription [1]. This was mainly because of technological advances made around mRNA sequencing, which enabled big data analyses with increased sensitivity of changes in mRNA expression. Two major advances over the past decade caused a shift in the focus from signal transduction to mRNA translation, one being the increase in throughput and sensitivity of proteomics technologies and the other the emergence of ribosome profiling [2], which emphasized the lack of correlation between mRNA levels and protein expression levels for many genes. mRNA translation is a highly regulated process: once mRNA is transcribed, it is bound by RNA-binding proteins (RBPs) in a highly specific and selective manner [3]; an additional layer of regulation is mediated by modifications of ribonucleotides in mRNA (epitranscriptomics), which modulated RBP–mRNA interactions. Together, these regulate mRNA processing, nuclear export, mRNA steady-state levels and translation. RBPs regulate the localization of mRNAs in the nucleus and cytoplasm, thereby determining mRNA translation in a temporal and spatial manner. Moreover, ribosomes, similar to RNA polymerases and proteasomes, have accessory proteins associated with them in a tissue and signal-specific manner [4], which lends mRNA translation the added layer of selectivity. Protein synthesis is a high energy-requiring process, and the translation machinery must respond quickly to changes in the environment to conserve cell energy. Thus, distinct mechanisms are in place that enable a fast response to extracellular signals, including coordinated changes in translation initiation by changing the components in the initiation complex; by shuttling mRNAs to cellular compartments protecting them from degradation, but excluding them from ribosomes; and by using nonprotein vehicles to regulate mRNAs, such as short-lived small noncoding mRNAs.

In different tissues, cells need to respond to distinct sets of cues. For example, in neurons, mRNA translation is localized to different cellular compartments. Ribosomes are located around the nucleus, along exons, and at nerve endings to enable supply on-demand of specific proteins required at these different cellular locations. Thus, to enable these diverse requirements and responses, mRNA translation has developed an elaborate, tissue-specific and mRNA selective regulatory systems.
TranslationLight
At Anima Biotech, we have developed TranslationLight, a high-content imaging-based technology that enables visualization of protein synthesis in cells using two fluorescently labeled tRNAs as Förster resonance energy transfer (FRET) pairs (Fig. 1a) [5,6]. The pair of tRNAs is selected from 46 human tRNAs to detect the translation of a single protein, over the background, by using our bioinformatics platform, CereBio. The signal:noise ratio observed using this technology is dependent on additional proteins, which use the same pair of tRNAs; on the occupancy of the selected di-codon by ribosomes; and on the rate of translation. Interestingly, for ~80% of human genes, a pair of codons can be identified to have sufficient signal:noise ratio. TranslationLight is amenable to high-throughput screening (HTS) in transfectable cells and

**FIGURE 1**
Anima’s TranslationLight Technology enables monitoring mRNA translation of a range of specific proteins inside cells. (a) Schematic representation of TranslationLight. Visualization of mRNA translation of a specific protein is possible via the use of a signature tRNA pair, two fluorescently labeled tRNAs, as a Förster resonance energy transfer (FRET) pair. Once two differently labeled tRNAs are sitting adjacent inside the ribosome during mRNA translation, a FRET signal is emitted, indicative of a specific protein translation event. (b) A selected pair of tRNAs, decoding a specific proline (Pro) and glycine (Gly) codon, are highly Collagen-I specific. (i) Quiescent WI38 fibroblasts were induced to translate large amounts of Collagen-I and transfected with a Pro-Gly-specific isoaacceptor tRNAs. Cells were fixed and FRET signals detected with an automated wide-field microscope (Operetta, Perkin-Elmer) (ii,iii). Introduction of COL1A1 small interfering (si)RNA to cells transfected with Collagen-I specific pair of tRNA resulted in a robust inhibition of the FRET signal (FRET signal, white; nucleus, blue). (c) Hit compounds decrease or increase Collagen-I translation. (i–iii) Show FRET signals in DMSO and two different hit compound-treated cells (FRET signal, light blue; nucleus, blue). (iv–vi) Accumulation of Collagen-I in WI38 cells, detected by anti-Collagen-I antibody. Compounds that decrease the FRET signal reduce the levels of collagen-I proteins, and compounds that increase the FRET signal enhance collagen-I accumulation in cells, demonstrating that Translationlight can identify compounds that reduce or induce translation in the same screening campaign (Collagen-I, green; nucleus, blue).
requires high-content microscopy coupled with high capacity for image and data analyses.

**Pulse labeling using puromycin**

Additional approaches that monitor mRNA translation [7] use short pulse labeling of nascent peptides by incorporation of puromycin. Puromycin-based proximity ligation assay (puro-PLA) allows in situ detection of newly synthesized proteins using two different primary antibodies that recognize the target protein and incorporated puromycin. A pair of oligonucleotide-labeled secondary antibodies then recognize and bind the two primary antibodies and, only if they are found in close proximity, a circular DNA template is then made by an exogenously added ligase. The DNA template is then amplified and detected by fluorescent probe hybridization for improved signal enhancement. This complex technique depends on the availability of highly specific antiprotein antibodies originating from species different than the anti-puromycin antibody, and numerous controls that need to be used to avoid nonspecific proximity detection. Moreover, the technique requires a short incubation time with puromycin, which renders it almost impossible to use in a high throughput manner, and the high cost per well and multiple wash steps prohibit its use in screens.

**Ribosome profiling**

Another widely used method to monitor translation in cells is ribosome profiling [8], which involves isolation of ribosome-bound mRNA by high-speed centrifugation, followed by deep sequencing of the ribosome-protected mRNA fragments. Using ribosome profiling, a snapshot of translated mRNA can be visualized, density of ribosomes per mRNA reading frame can be analyzed, and novel and alternative protein products can be discovered. Given the complex experimental set-up and need for rapid translation inhibition to prevent alteration in the local distribution of ribosomes on the mRNA, biases can be introduced during sample handling. Unlike TranslationLight and puro-PLA, which are single cell-based assays that enable detection of single-cell differences in target protein translation, ribosome profiling is done in bulk on millions of cells and, thus, gives a population-based translation signature. Moreover, this technology is not amenable to HTS. Together, the three techniques give different viewpoints and orthogonal information on target protein translation.

**Monitoring Collagen-I in idiopathic pulmonary fibrosis**

Idiopathic pulmonary fibrosis (IPF) is a progressive and usually lethal interstitial lung disease of unknown etiology characterized by varying degrees of inflammation and fibrosis in the lung parenchyma. The fibrotic response is generally considered an irreversible process and is characterized by an increase in resident fibroblasts, which leads to profound and complex changes in extracellular matrix (ECM) turnover, and increased deposition of Collagen type I (Collagen-I). Indeed, an imbalance in Collagen-I accumulation in the local lung microenvironment appears to be of central importance in the pathogenesis of the fibrotic component of IPF. Given that Collagen-I is not a druggable protein, there were and are many attempts to indirectly inhibit collagen deposition in fibrotic lungs. Using our technology, we directly monitored the translation of Collagen-I and identified compounds that directly modulate its deregulated expression in IPF.

To monitor Collagen-I (encoding by COL1A1) translation, a pair of proline and glycine isocodon tRNAs was selected and used to detect its translation in the human lung fibroblast cell line WI38. There are nine potential pairs of tRNAs isocodonets that can decode proline and glycine codons in the human transcriptome (three codons for proline and three for glycine). Nonetheless, human Collagen-I preferentially uses one pair, found 106 times out of the 202 proline–glycine codon pairs in its mRNA. Thus, the selected tRNAs were isolated and labeled as FRET pairs (Cy3 and Cy5) [5] and transfected into WI38 fibroblasts. Treatment of tRNA-transfected WI38 fibroblasts with a Collagen-I induction cocktail resulted in an increase of Collagen-I-selective FRET signal relative to non-induced cells, reflecting the increase in Collagen-I protein synthesis in induced fibroblasts (Fig. 1bI versus bII). When mRNA against COL1A1 was transfected into cells together with labeled di-tRNAs Pro-Gly, the FRET signal emitted from ribosomes was reduced by >90%, showing that the FRET signal was emitted by ribosomes translating Collagen-I (Fig. 1bII versus bII).

**Experimental approach**

We used this FRET-tRNA-specific Collagen-I translation assay to screen in a high throughput manner a small-molecule, drug-like, diverse library of 100,000 compounds. WI38 cells were plated in 384-well plates and transfected with a Pro-Gly tRNA pair mixed with transfection reagent. Plates were incubated at 37 °C, 5% CO2 overnight. The next day, a Collagen-I induction cocktail was added to each well, followed by compounds to a final concentration of 30 μM. Cells were incubated with compounds for 24 h at 37 °C before fixation with 4% paraformaldehyde (PFA). Cell nuclei were stained with DAPI and slides imaged with Operetta high content microscope (Perkin Elmer). Images were automatically uploaded to a cloud-based architecture designed in house. Data were analyzed in an individual cell-based manner, generating ~90 image-based features, which correlated to different FRET-based phenotypes. These data were analyzed using our big data, supervised and non-supervised, algorithms. A uniform control cell population was generated per plate, based on control wells equally distributed in each of the screen plates. Cells from compound treated wells were compared with this control cell population, resulting in robust statistical analyses and an unbiased hit detection methodology.

Validation of hit activity was done by retesting screen hits in the primary. Pro-Gly tRNA pair assay and by measuring Collagen-I protein accumulation by immunofluorescence. Global translation inhibitors were eliminated by counter assays, measuring global translation by using TranslationLight with all tRNAs as FRET pairs and by metabolic labeling using fluorescent methionine. Compounds that either decreased or increased Collagen-I translation were identified (Fig. 1cI and cII, respectively), showing that TranslationLight offers an unbiased methodology for hit finding and is able to identify compounds that increase or decrease protein synthesis: an increase in FRET signal by compound treatment reflects an increase in protein mRNA translation, whereas a decrease in FRET signal is indicative of lower translation of target protein (Fig. 1c, compare upper with lower images). An additional
Tissue-selective regulation of mRNA translation: Collagen-I accumulation is reduced only in lung fibroblasts and not in skin or liver fibroblasts. (a) ANI-21 significantly reduced Collagen-I accumulation in a dose-dependent manner in WI38 lung fibroblasts. (i-iii) Collagen-I levels in either un-induced (quiescent) or induced WI38 cells following DMSO (control) addition. (iii-vi) Reduction of Collagen-I in induced WI-38 cells following treatment with ANI-21, 3, 30, and 300 nM (immunofluorescence: anti-Collagen-I, green; nucleus, blue). (b) Graphical representation of the dose-dependent reduction of collagen-I by ANI-21. Data were generated from anti-Collagen-I stained cells. (c) ANI-21 selectively reduced Collagen-I in WI38 fibroblasts (i) but not in LX-2 (liver (ii)) or normal human derived fibroblasts (HDF) (skin (iii)) (immunofluorescence: anti-Collagen-I, green; nucleus, blue). (d) Graphical representation of the EC_{50} of ANI-21 in the different cells. Data were generated from anti-Collagen-I stained cells.
benefit of TranslationLight is the single cell-based analysis, which is sensitive to small changes in cell populations responding to translation regulation by an exogenous signal, or compound.

**Results**

We identified three distinct chemical scaffolds that specifically decreased Collagen-I translation without affecting global mRNA translation. Compound optimization was driven by using panels of translation regulation assays and monitoring Collagen-I protein and mRNA in target cells. Compound activity was optimized to two-digit nanomolar activity in reduction of Collagen-I translation in cells (Fig. 2a and 2b). In parallel, mRNA transcription to translation mode of action studies and a target identification campaign were conducted.

COL1A1 mRNA translation is controlled by different post-transcriptional regulatory pathways in a tissue-selective manner [9]. Indeed, several adaptor proteins and RBPs bind to 5′- and 3′- untranslated regions of Collagen-I mRNA and tether it away from active ribosomes (polysomes) or recruit it to polysomes (e.g., LARP6 and p180) [10]. To study the tissue-selective activity of our compounds, potent compounds from each of the series were tested in human fibroblasts derived from lung, liver, skin, and bone (osteoblasts) (Fig. 2b, and Fig. S1 in the supplemental information online). Compound ANI-21 reduced Collagen-I accumulation in a dose-dependent manner in lung-derived fibroblasts (Fig. 2a,b, EC50 = 0.027 nM), but showed a 3-log lower potency in human liver fibroblasts (LX-2 stellate cells; Fig. 2c,d). ANI-21 did not reduce Collagen-I translation in primary skin fibroblasts and osteoblasts (Fig. 2c,d, and Fig. S1 in the supplemental information online). This unique profile of tissue selectivity gives our compounds a safety advantage because the compounds will only affect Collagen-I production in lung, without affecting wound-healing process in skin and liver, and will not affect bone formation.

We also developed Compass, a collection of technologies, assays, and bioinformatics algorithms focused on the biology of mRNA maturation, transport, localization, and translation. These are used to rapidly characterize the mode of action of compounds and efficiently identify the of the target-mediating effect of the compounds. Towards this end, the kinetics of the effect of the compounds on Collagen-I mRNA and protein were studied in a dose and time-dependent manner in fibroblasts derived from different tissue origins (Fig. 3 and Fig. S1 in the supplemental information online). In lung fibroblasts, COL1A1 mRNA was localized to the cytoplasm in both quiescent and induced cells (Fig. 3a). Transcription sites were detected in the nucleus and, upon induction, the percentage of cells in the cell population exhibiting COL1A1 transcription sites increased, whereas levels of COL1A1 mRNA in the cytoplasm were slightly increased. This increase in transcription was not correlated with the robust increase observed in Collagen-I protein levels (Fig. 3a1 versus aii), indicating that COL1A1 mRNA accumulates in the cytoplasm, tethered away from translating ribosomes. Upon induction signals, COL1A1 mRNA is transported to ER-localized ribosome to be translated. The effect of the Collagen-I induction cocktail on transcription and mRNA localization was distinct in fibroblasts derived from different tissues (Fig. S2 in the supplemental information online), hinting at the distinct mode of regulation of Collagen-I induction in response to injury in different tissues. Compound ANI-21 did not affect either transcription sites or mRNA levels in the cytoplasm (Fig. 3bii), whereas it completely inhibited protein accumulation (Fig. 3bi). Thus, ANI-21 might regulate Collagen-I mRNA recruitment to ribosomes in the cytoplasm, thereby inhibiting its translation.

The effect of ANI-21 on gene and protein expression in WI38 fibroblasts was evaluated by RNA sequencing and mass spectrometry analysis, respectively. Only small differences were observed on gene and protein expression after 24 h of compound incubation (Fig. 3ci and cii, respectively). The minor changes observed in protein expression in lung fibroblasts in response to ANI-21 were well correlated with other studies we have conducted (immuno-fluorescence analyses of TIMP1 and alpha smooth muscle actin, Fig. 3d and data not shown, respectively; and metabolic labeling, data not shown), showing that the compound selectively regulates Collagen-I translation without affecting general mRNA translation. Most of the changes observed by ANI-21, on both RNA and protein levels, were in the reduction of expression of genes and proteins involved in fibroblast differentiation into myofibroblasts, regulating collagen maturation and matrix deposition (Fig. 3ci, marked by orange circles). Indeed, one of the markers of fibroblast to myofibroblasts differentiation, TIMP1, was reduced by ANI-21 in WI38, as assessed by immunofluorescence assay (Fig. 3d). Proteins and RNAs implicated in the regulation of pre-RNA processing were additionally differentially expressed. Thus, ANI-21 inhibited the progression of resting fibroblasts into specialized collagen producing cells by specifically affecting collagen-I mRNA accessibility to ribosomes, resulting in inhibition of collagen translation.

To check whether ANI-21 antifibrotic activity was translated from cell-based assays to efficacy in animal models of pulmonary fibrosis, we used the widely used Bleomycin (BLM)-induced animal model in mice [11]. As reference substances, nintedanib, a tyrosine kinase inhibitor approved for IPF, and GLPG1690, an autotxin inhibitor found in two Phase III studies in IPF, were selected. First, we compared the effect of ANI-21 to the two reference substances in the Collagen-I deposition assay in WI38 fibroblasts (Fig. 4a). ANI-21 completely inhibited Collagen-I accumulation (Fig. 4a, ANI-21 = 300 nM), reducing levels back to those detected in non-induced cells (Fig. 4a, compare the images of ANI-21-treated to the DMSO, uninduced cells). Both nintedanib (1 μM) and GLPG1690 (1 μM) showed a modest decrease in Collagen-I accumulation (Fig. 4a).

ANI-21 (3 and 10 mg/kg), or the corresponding vehicle (DMSO:10% solutol:saline, 1:1:8, V/V/V/) were dosed twice daily (b.i.d.) for 21 days (from day 0 to day 21) by intraperitoneal administration (i.p.) (Fig. 4b,c). ANI-21 doses were selected based on pharmacokinetics (PK) analysis in mice, which indicated adequate compound exposure in plasma and lung (Fig. S3 in the supplemental information online). Nintedanib (60 mg/kg, oral) was administered once daily, and GLPG1690 (15 mg/kg, i.p.) twice daily, for 21 days. Schedule and doses for reference substances were selected based on reported efficacies in bleomycin models [12,13]. The efficacy of ANI-21, nintedanib, and GLPG1690 was evaluated based on the assessment of histopathological changes in lung architecture using the modified Ashcroft score [14]. Intranasal application of BLM induced diffuse epithelial injury, pulmonary inflammation, and fibrosis as observed in the vehicle control group (Fig. 4b). Within 21 days, ANI-21 dose-dependently and
ANI-21 regulates Collagen-I translation by inhibiting mRNA recruitment to ribosomes. (a) Collagen-I mRNA and protein levels in un-induced (quiescent) and induced WI-38 cells. (i) Collagen-I protein accumulation following induction (immunofluorescence: anti-Collagen-I, green; nucleus, blue). (ii) Fluorescent in situ hybridization with a COL1A1-specific probe (Col1A1, red; nucleus, blue). (b) Collagen-I mRNA and protein levels in induced cells treated with DMSO or ANI-21. (i) Total reduction in Collagen-I protein accumulation following induction (Collagen-I, green; nucleus, blue). (ii) Similar COL1A1 mRNA levels of induced cells treated with DMSO or ANI-21 (COL1A1, red; nucleus, blue). (c) ANI-21 induces small changes in mRNA and protein levels. (i) Volcano plots of differentially expressed genes between ANI-21 treated and DMSO-treated induced for 24 h (RNA sequencing). (ii) Volcano plots of differentially expressed proteins in ANI-21 treated and DMSO-treated induced for 24 h (mass spectrometry analysis). TIMP1 is indicated on the volcano plot. (d) Images of WI38 cells stained for the fibrotic marker TIMP1. Induced WI38 cells treated with ANI-21 demonstrated reduced TIMP1 expression compared with DMSO-treated cells (TIMP1, red; nucleus, blue).
ANI-21 demonstrates a robust antifibrotic effect, in a bleomycin-induced lung fibrosis model, similar to inhibition observed in cells. (a) Effect of ANI-21 in a Collagen-I deposition assay in WI38 cells. ANI-21 reduced Collagen-I accumulation to levels of un-induced cells, whereas the two reference compounds, nintedanib and GLPG1690, showed a more modest decrease (immunofluorescence: anti-Collagen-I, orange; nucleus, blue). (b) Effect of ANI-21 in a bleomycin (BLM)-induced IPF model assessed by Masson’s trichrome staining. Lung fibrosis was induced in mice by two intranasal instillations of BLM on days 0 and 4. Mice treated with ANI-21 for 21 days showed reduced collagen accumulation and immune cell infiltration, as well as improved tissue architecture compared with reference substances. (d) Graphic representation of BLM model, showing the modified Ashcroft score of two doses of ANI-21 (3 and 10 mg/kg), nintedanib (60 mg/kg) and GLPG1690 (15 mg/kg). Results were expressed as mean ± SEM. Statistical analysis was performed using one-way or two-way ANOVA followed, if significant, by post-hoc Dunnett’s test (**P < 0.01; ***P < 0.001). (d) Schematic representation of the target space in mRNA translation regulation.
highly statistically significantly reduced the Ashcroft score compared with the vehicle-treated group (Fig. 4b,c). Treatment with reference compounds resulted in a trend of reduction in the Ashcroft score, but did not reach statistical significance. Thus, ANI-21 activity in the reduction of Collagen-I translation and accumulation in cell-based assays translated to efficacy in BLM-induced lung fibrosis, resulting in similar levels of inhibition of collagen-I deposition.

**mRNA translation and ANI-21**

We have optimized the drug-like properties of ANI-21 to enable oral administration toward the development of an oral drug candidate for IPF. ANI-21 acts by inhibiting the induction of collagen-I mRNA translation in activated fibroblasts, thereby tapping into a novel and diverse target space, regulation of mRNA translation. mRNA, as the molecule that relays instructions from DNA to the translation machinery, undergoes a range of processing, localization, and stabilization processes, which have evolved to selectively and differentially regulate translation in response to intracellular and extracellular cues. Multiple layers of regulation are found after mRNA transcription, with nucleotide processing and protein binding occurring co-transcriptionally. Pre-RNA splicing, modification of ribonucleotides [15], and modifications of 5'- and 3' -UTRs dictate mRNA-protein association that determines mRNA nuclear transport dynamics and mRNA half-life (Fig. 4d). There are hundreds of RBPs [16] that have an integral part in these regulatory events. mRNA selectivity and tissue specificity are conferred by tissue-specific expression of RBPs [17] and their regulatory enzymes, enabling differential regulation of the same mRNA in different tissue. Moreover, these RBPs are part of multiprotein complexes that regulate the cytoplasmic localization of mRNAs, thereby determining the time and rate of mRNA translation. In addition, diverse families of enzymes and accessory proteins modify and bind to ribosomes, resulting in different populations of ribosomes that are responsible for the translation of specific mRNA, or a group of mRNAs that occur in a shared pathway or complex. The mode of action of ANI-21 is currently in the final stages of characterization. However, it appears that it indirectly affects collagen-I translation initiation by regulating the cytoplasmic localization of Collagen-I mRNA and does not directly affect Collagen-I mRNA translation by ribosomes.

**Concluding remarks**

In viewing the kinetics of mRNA translation and building a platform that focuses on the identification of mRNA translation regulation and target space, TranslationLight is a high-throughput method that enables the identification of small molecules that regulate the activity of novel targets in a vast target space that regulate mRNA translation into proteins.

**Appendix A. Supplementary data**

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.drudis.2020.11.017.

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**References**


