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Potent and Selective Knockdown of Tyrosine Kinase 2 by Antisense Oligonucleotides

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ABSTRACT

Tyrosine kinase 2 (TYK2) (1) is a member of the JAK family of nonreceptor tyrosine kinase, together with JAK1, JAK2, and JAK3. JAKs are important signaling mediators of many proinflammatory cytokines and represent compelling pharmacological targets for autoimmune and inflammatory diseases. Pan-acting small-molecule JAK inhibitors were approved for the treatment of rheumatoid arthritis and ulcerative colitis. However, their limited selectivity among JAK members have led to undesirable side effects, driving a search toward specific JAK inhibitors. Recently, TYK2 has emerged as a target of choice for the treatment of autoimmune diseases and severe COVID-19 with an optimum balance between efficacy and safety, based on observations from human genetics studies and clinical outcomes of several agents targeting cytokine pathways for which TYK2 plays an essential role. In this article, we address selective targeting of TYK2 from the genetic sequence space through development of antisense oligonucleotides (ASOs) against TYK2 mRNA. Potent ASO candidates were identified from the screening of over 200 ASOs using locked nucleic acid gapmer design. The lead ASOs exhibited potent and selective knockdown of TYK2 mRNA and protein across a panel of model human cell lines in a dose-dependent manner, showing no reduction in the mRNA and protein expression levels of other JAK paralogs. In agreement with the depletion of TYK2 proteins, several TYK2-mediated cytokine signaling pathways, including IFN-α and IL-12, were inhibited upon ASO treatment. Our results established the TYK2 ASOs as investigational tool compound and potential therapeutic agent for the treatment of autoimmune diseases and severe COVID-19. ImmunoHorizons, 2021, 5: 70–80.

INTRODUCTION

Tyrosine kinase 2 (TYK2) (1) is a member of the JAK family of nonreceptor tyrosine kinase (2), together with JAK1, JAK2, and JAK3. JAKs associate with various cytokine receptors at the cytoplasmic side, playing a crucial role as signaling mediators. Upon extracellular cytokine binding to the receptors, the bound JAK proteins phosphorylate each other as well as the receptors, which then serve as anchor points for transcription factors known as STAT. The STAT members are in turn activated by tyrosine phosphorylation and undergo subsequent dimerization and translocation to the nucleus to propagate downstream gene signaling (2). Importantly, JAK kinases are critical signal transducers for numerous proinflammatory cytokines (3) and thus represent compelling pharmacological targets for the treatment of autoimmune and inflammatory diseases (4). As such, intensive efforts have been directed toward the development of small-molecule JAK inhibitors (5, 6), with the first approval for the treatment of rheumatoid arthritis by such agents being granted by the U.S. Food and Drug Administration in 2012 (7). However, this and other
first-generation JAK inhibitors are pan-acting with limited selectivity between different JAK paralogs. Their expansion toward additional autoimmune indications has been hampered by unfavorable safety profiles at higher therapeutic doses, most notably through inhibition of JAK2, a critical signal transducer in hematopoiesis (8). As such, present drug development efforts focus on the selective targeting of individual JAK members to achieve robust clinical efficacy while minimizing undesirable side effects (4, 5). This presents a significant challenge, owing to the high homology among the various JAK paralogs (5).

Recently, selective TYK2 targeting has emerged as an attractive treatment option for a range of autoimmune diseases. TYK2 plays an essential role in the signaling pathways of multiple cytokines (9–11), including type I/III IFN, IL-12, IL-23, and IL-10. Human genome-wide association studies (12–14) revealed that deactivating TYK2 variants could provide protection against a broad range of autoimmune conditions, including psoriasis (PSO), rheumatoid arthritis, systemic lupus erythematosus (SLE), ankylosing spondylitis, ulcerative colitis, Crohn's disease, type 1 diabetes, and multiple sclerosis. Association of TYK2 with these conditions was also supported by findings from other studies (15–20). Furthermore, drug agents targeting IFN-α and IL-12/IL-23 signaling, all of which share TYK2 as a common signaling transducer, have shown clinical efficacy in various autoimmune conditions (21, 22). Among the JAK family members, TYK2 would represent a safer target of choice, as JAK1-knockout (perinatal lethal), JAK2-knockout (embryonic lethal), and JAK3-knockout (SCID) mice displayed fatal/critical developmental defects (10, 23), whereas TYK2-knockout mice showed relatively milder deficiencies (9, 23, 24) (susceptibility to mycobacterial, viral, and fungal infections). There are several TYK2-selective inhibitors undergoing active clinical development (6, 25). Different strategies were used in the design of the inhibitors, including active-site inhibition, simultaneous inhibition of TYK2/JAK1 (26), and allosteric binding of pseudokinase domain (27, 28). Early readouts from the clinical trials are encouraging (29, 30), although longer-term efficacy and safety of these agents are yet to be determined (31). Additional TYK2-targeting agents that use different modes of action will expand on the drug development repertoire and are thus highly desirable.

With the ongoing COVID-19 pandemic around the world, effective treatment options for COVID-19 are urgently needed. In severe COVID-19 cases, immune overactivation leading to cytokine release syndrome can be fatal. As such, pan-JAK inhibitors including ruxolitinib and baricitinib, both of which inhibit JAK1 and JAK2, were brought forward as potential treatment for severe COVID-19. However, ruxolitinib failed to show clinical benefit in a recently reported phase III study (https://www.novartis.com/news/media-releases/novartis-provides-update-ruxcovid-study-ruxolitinib-hospitalized-patients-covid-19). Although baricitinib received emergency use authorization (https://www.fda.gov/news-events/press-announcements/coronavirus-covid-19-update-fda-authorizes-drug-combination-treatment-covid-19) by the U.S. Food and Drug Administration for use in combination with remdesivir, outcomes from additional clinical trials still await. A just-released genome-wide association study of critically ill COVID-19 patients (32) has linked high TYK2 expression to severe COVID-19, pointing to the selective targeting of TYK2 as a potential treatment for these severe cases.

RNA therapeutics including antisense oligonucleotides (ASOs) and small-interfering RNAs represent an emerging drug modality that have seen recent clinical breakthrough successes (33). These agents operate on a genetic basis through complementary hybridization to their intended RNA targets (34), thus allowing the rapid development of lead compounds against any gene target of choice. In this study, we approach selective targeting of TYK2 from the genetic sequence space through the development of ASOs against TYK2 mRNA. Potent ASO candidates were identified from a screening of over 200 ASOs using locked nucleic acid (35, 36) (LNA) chemistry. The lead ASOs exhibited potent and selective knockdown of TYK2 mRNA and protein across a panel of model cell lines in a dose-dependent manner, showing no reduction in the mRNA and protein expression levels of other JAK paralogs. Consistent with the depletion of TYK2 proteins, several TYK2-dependent cytokine signaling pathways, including IFN-α and IL-12, were inhibited after pretreatment with the ASOs. Our results established the TYK2 ASOs as investigational tool compound and potential therapeutic agent for the treatment of autoimmune diseases and severe COVID-19.

MATERIALS AND METHODS

ASO design, synthesis, and purification

Unless otherwise specified, 16-nt fully phosphorothioate (37) (PS)-modified ASOs incorporating LNA (35, 36) in a 3-10-3 gapmer (38) configuration were designed to be complementary to different segments of TYK2 mRNA (Refseq identifier NM_003331.4). All ASOs were synthesized in-house with an ABI 394 DNA/RNA synthesizer on Glen UnySupport (Glen Research) using standard phosphoramidite chemistry. LNA phosphoramidites were purchased from either Exiqon or Sigma-Aldrich or synthesized from 3'-hydroxyl precursors (Rasayan). Pheny lacetyl disulfide (ChemGenes Corporation) was used as the sulfurizing reagent. The ASOs were cleaved from solid support and deprotected with concentrated aqueous ammonia at 55°C for 16 h and subsequently purified using Poly-Pak II cartridges (Glen Research) according to the manufacturer’s protocol. The ASOs were desalted using Glen Pak 2.5 desalting column (Glen Research) and dried by lyophilization. The dried ASOs were resuspended with 20 mM potassium phosphate (pH 7.0) into 100 μM stock solutions and diluted with cell culture medium before use. All ASOs were characterized by Jeol SpiralTOF MALDI-TOF mass spectrometer.

Cell culture and reagents

Jurkat acute T cell leukemia, MOLT-4 acute lymphoblastic leukemia, K-562 chronic myelogenous leukemia, A-431 epidermoid carcinoma, and HeLa adenocarcinoma cells were purchased from American Type Culture Collection. Karpas-299 non-Hodgkin large cell lymphoma and HDLM-2 Hodgkin lymphoma...
cells were purchased from Sigma-Aldrich and Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), respectively. NK-S1 NK/T cell lymphoma cells were generously provided by Prof. W.J. Chng (Cancer Science Institute of Singapore). Suspension cells were typically cultured in RPMI 1640 medium (Thermo Fisher Scientific), whereas adherent cells were cultured in DMEM (Thermo Fisher Scientific), all supplemented with 10–20% FBS (Thermo Fisher Scientific). NK-S1 cell was cultured in DMEM supplemented with 10% FBS and 10% equine serum (Sigma-Aldrich). All cells were grown in a 5% CO₂ humidified incubator at 37°C.

**ASO treatment**

Cells were seeded out on 12- or 24-well plates (Corning) and grown for 24 h prior to being treated with ASO. ASOs were diluted with fresh culture media and added directly into treatment wells to attain the desired concentrations, without the use of any transfection reagents. For time-point measurements, cells were harvested at various intervals (8 and 16 h and 1, 2, 3, and 4 d) post-ASO treatment. For all other experiments, including RNA/protein level measurements and cytokine stimulation assays, cells were harvested 2 or 3 d post-ASO treatment.

**Quantitative reverse transcription PCR**

Total RNA from each culture well was extracted by successive addition of 1) TRIzol reagent (Thermo Fisher Scientific) for cell lysis, 2) chloroform for phase separation, and 3) isopropanol for RNA precipitation. The RNA pellet was washed with ethanol and allowed to air dry and subsequently resuspended in RNase-free water. Quantiﬁcation of the RNA was performed using Nanodrop 2000 (Thermo Fisher Scientific). The RNA was then reverse transcribed with M-MLV reverse transcriptase (Promega) following the manufacturer’s protocol. Quantitative PCR of the cDNA was performed using either SYBR Green- or probe-based master mix (Bio-Rad) on a Bio-Rad CFX96 real-time PCR detection system. The 2⁻ΔΔthreshold cycle method (39) was employed to determine expression level of the target genes using GAPDH as a reference.

**Primer and probe sequences**

The primer pairs used in ASO screening and JAK selectivity measurement are as follows: **TYK2**: (forward) 5’-GGA GAG GGG TTC TGG TAG CA-3’, (reverse) 5’-ATG TCC CGG AAG TCA CAG AAG-3’; **JAK1**: (forward) 5’-CTT TGG CCT GTA TGA CGA GAA C-3’, (reverse) 5’-ACC TCA TCC GTG AGT CGA GC-3’; **JAK2**: (forward) 5’-ATC CAC CCA ACC ATG TCT TCC-3’, (reverse) 5’-ATT CCA TGC CGA TAG GCT CTG-3’; **JAK3**: (forward) 5’-CTT GAT CGT CCT GGA CGA AG-3’, (reverse) 5’-GCA GGG ATC TTG TGA AAT GTC AT-3’; and **GAPDH** (reference): (forward) 5’-CTG GGC TAC ACT GAG CAC C-3’, (reverse) 5’-AAG TGG TCG TTG AGG GCA ATG-3’, (probe) 5’-TCT CCT CTG ACT TCA GCG ACA CCC-3’. All primers and probes were purchased from Integrated DNA Technologies.

**Western blot and cytokine stimulation**

Cells were harvested and lysed in mammalian protein extraction reagent (Thermo Fisher Scientific) containing protease and phosphatase inhibitor mixture (Sigma-Aldrich) following the manufacturer’s protocol. Protein samples were quantiﬁed by BCA protein assay kit (Thermo Fisher Scientiﬁc). Approximately 10–30 μg of total protein was loaded and run on 10% SDS-PAGE with XT MOPS running buffer (Bio-Rad) and subsequently transferred onto a polyvinylidene diﬂuoride membrane (GE Healthcare). The membrane was blocked by 5% nonfat dry milk (Bio-Rad) in TBST for 1 h at room temperature, followed by 5-min wash with TBST three times over. The membrane was then probed with primary Abs against the target proteins (TYK2: no. 9312; JAK1: no. 3344; JAK2: no. 3230; JAK3: no. 8827; STAT1: no. 9172; pSTAT1-Y701: no. 7649; STAT3: no. 4904; pSTAT3-Y705: no. 9145; pSTAT4-Y693: no. 4134; and GAPDH: no. 5174; Cell Signaling Technology) in the recommended buffers, either at room temperature for 1–2 h or at 4°C overnight. After washing for three times, the membrane was incubated with HRP-labeled secondary Abs (no. 7074; Cell Signaling Technology) at 1:5000–1:2000 dilution in the blocking buffer at room temperature for 1 h. The membrane was washed for three times, followed by incubation with ECL substrate (Bio-Rad) according to the manufacturer’s protocol. Finally, the target bands were visualized and captured on Amersham Imager 680 (GE Healthcare) or Gel ChemiDoc system (Bio-Rad). In cases in which multiple JAKs had to be imaged, the order of Ab incubation, stripping, and reprobing were as such: TYK2 followed by JAK2 and JAK3 followed by JAK1. The membrane was stripped using a stripping buffer (100 mM 2-ME, 2% SDS, 62.5 mM Tris-HCl, pH 6.7) at a temperature of 50°C for 30 min with gentle agitation, followed by six-time washing of 5 min each by TBST, before proceeding to reblock the membrane and reprobe with Abs.

For cytokine stimulation assays, the cells lines were seeded for 24 h, followed by ASO treatment for a further 3 d before being subjected to stimulation by the respective cytokines as follows: IFN-α (Genscript) at 30 ng/ml for 30 min, IL-12 (Sigma-Aldrich) at 20 ng/ml for 1 h, IL-10 (Genscript) at 100 ng/ml for 1 h, and IL-6 (Genscript) at 100 ng/ml for 1 h.

**ELISA measurement of IFN-γ secretion**

IFN-γ secreted by NK-S1 cell upon IL-12 stimulation was measured by Quantikine ELISA kit (R&D Systems) following the manufacturer’s protocols. The cells were treated with 10 μM ASO-J for 3 d before stimulation with 20 ng/ml IL-12 (Sigma-Aldrich) for 12 h. Following stimulation, 100 μl of cell culture was deposited into individual ELISA wells and incubated at room temperature for 2 h to allow IFN-γ binding to precoated Abs. The wells were rinsed after removal of media, followed by 2-h incubation with 100 μl of HRP-labeled secondary Abs. Unbound Abs were washed away.
before HRP substrate was added to the wells for color development over 30 min. The reaction was quenched with 0.2 M H₂SO₄, and OD₄₅₀ for individual wells was recorded by μQuant microplate reader (BioTek).

Cytotoxicity measurements of ASOs

Cytotoxicity of ASOs was measured based on their activation of cellular caspase 3/7. Ten thousand HeLa cells were seeded in 100 μl of DMEM into 96-well plates for 24 h before being transfected with 100 nM or 1 μM ASOs using Lipofectamine RNAiMAX (Thermo Fisher Scientific) for an additional 24 h. Subsequently, 100 μl of Caspase Glo 3/7 Assay (Promega) was added to each well, and the plates were kept in the dark for 30 min. The resulting luminescence was recorded by a TECAN Infinite M200 plate reader and background subtracted according to the manufacturer’s protocol. Relative caspase activation was calculated as the ratio of luminescence reading between the treated samples and the untreated control, of which a high value indicates high cytotoxicity of the ASO.

RESULTS

Potent knockdown of TYK2 mRNA and protein by TYK2-specific ASOs

To identify potent TYK2 ASO candidates, we performed screening on over 200 TYK2-specific ASOs for their potential to induce RNase H-mediated mRNA cleavage in Jurkat leukemia cells, a T-lymphocyte model cell line. Unless otherwise specified, the ASO configuration comprises 16-nt fully PS (37)-modified gapmer (38) with a middle 10-nt DNA stretch flanked on both ends by 3-nt LNA (35, 36) segments (i.e., a wing-gap-wing composition of 3-10-3). The high potency of LNA gapmers enable cellular mRNA knockdown to be achieved in vitro simply by free incubation with ASOs [otherwise termed as free uptake (40, 41) or gymnosis (42) in the literature] without the use of any delivery agent. Five selected ASOs (ASO-1–5) demonstrated clear dose-response knockdown of TYK2 mRNA (Fig. 1A) with half maximal IC₅₀ values in the range of ~90–240 nM following 72 h of ASO treatment. Potency of a selected ASO, ASO-1, was further

FIGURE 1. ASO knockdown of TYK2 mRNA and protein.

(A) TYK2 mRNA levels, normalized against GAPDH, of Jurkat cell line after incubation with varying doses of ASOs for 72 h. n = 3, from three independent experiments. IC₅₀ values of the ASOs are shown in the legend. (B) TYK2 mRNA levels, normalized against GAPDH, of different cell lines (Jurkat, K-562, Karpas-299, MOLT-4, A-431, and HDLM-2) after incubation with varying doses of ASO-1 for 72 h. n = 3 for Jurkat, K-562, Karpas-299, and MOLT-4; n = 2 for A-431 and HDLM-2, from at least two independent experiments. The IC₅₀ values across the cell lines are shown in the legend. (C) TYK2 mRNA levels, normalized against GAPDH, of different cell lines (Jurkat, Karpas-299, and MOLT-4) after incubation with 0.5 μM ASO-1 or the control oligonucleotide (oligo) ASO-ctrl across varying time points from 8 h to 4 d. n = 2, from two independent experiments. Karpas-299 showed a rapid depletion of TYK2 mRNA upon ASO-1 treatment, whereas MOLT-4 showed a slower rate of depletion. (D) Western blot showing the dose-dependent knockdown of TYK2 protein in Jurkat cell line after incubation with varying doses of ASO-1 for 72 h. n = 3, from three independent experiments. TYK2 protein levels were not affected by the control oligo ASO-ctrl.

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measured across different cell lines, including suspension (Karpas-299 non-Hodgkin lymphoma, MOLT-4 acute lymphoblastic leukemia, K-562 chronic myelogenous leukemia, and HDLM-2 Hodgkin lymphoma) and adherent (A-431 epidermoid carcinoma) cells, giving rise to IC50 values ranging from 13 to 155 nM (Fig. 1B). Time-course measurement of RNA levels following ASO-1 treatment was performed across different cell lines (Fig. 1C); maximal knockdown was generally observed after 24–48 h and maintained thereafter for at least 4 d posttreatment with no replacement of media and ASO. In the same experiment, a control oligonucleotide (oligo) comprising the same LNA gapmer configuration (ASO-ctrl) did not lead to reduction in TYK2 mRNA level, thereby confirming the on-target effect of the TYK2-specific ASOs.

TYK2 protein levels in Jurkat cell were measured after treatment with varying concentrations of ASO-1 for 72 h. Near-complete depletion of the TYK2 protein was observed at an ASO concentration of 1 μM (Fig. 1D). At the same dosage, the control oligo ASO-ctrl did not affect the level of TYK2 protein, further validating the on-target effect of the TYK2-specific ASOs. The dose-dependent experiments were repeated on various suspension cell lines and showed the near-complete depletion of TYK2 across these cell lines upon ASO-1 treatment, even at the lower dosage of 100 nM (Fig. 2). Time-course measurement of protein levels following incubation of Jurkat cell with 1 μM of ASO-1 (Supplemental Fig. 1A) showed protein knockdown as soon as 24 h posttreatment, with progressively more knockdown observed at 48 and 72 h posttreatment.

Selective TYK2 knockdown by ASO-1 with no perturbation on other JAK paralogs
To be an effective therapeutic option, the ASOs should be specific for TYK2 while selective against all other JAK family members. Toward that end, JAK1, JAK2, and JAK3 protein levels in various cell lines were not reduced by treatment with ASO-1, even at dosages whereby the TYK2 protein was completely depleted (Fig. 2, Supplemental Fig. 1A). The TYK2 specificity of ASO-1 was also validated in the context of mRNA, for which only TYK2 mRNA knockdown was observed, whereas JAK1, JAK2, and JAK3 mRNA levels remained largely unchanged when 1 μM ASO-1 was incubated across different cell lines (Fig. 3). High TYK2 selectivity was similarly observed for ASO2–5, as JAK1, JAK2, and JAK3 mRNA levels in Jurkat cell remained largely constant following ASO treatment (Supplemental Fig. 1B).

ASO-1 inhibition of TYK2-mediated cytokine signaling pathways
Next, we evaluated the effects of TYK2 knockdown on various TYK2-associated cytokine signaling pathways (10, 11) (i.e., IFN-α, IL-12, IL-10, and IL-6) across different cell lines in vitro. For each signaling pathway, the cell lines were pretreated with ASO-1 for 3 d prior to being subjected to cytokine stimulation. Phosphorylation status of the relevant STAT members was then quantified by

FIGURE 2. Selective knockdown of TYK2 protein by ASO-1.
(A) Western blot showing the protein levels of all four JAKs in Jurkat and Karpas-299 cell lines after incubation with varying doses of ASO-1 for 72 h. (B) Western blot showing the protein levels of JAK1, JAK3, and TYK2 in MOLT-4 and K-562 cell lines after incubation with varying doses of ASO-1 for 72 h. Experiment was performed on four independent cell lines (n = 4). Potential slight increase of JAK1 and JAK2 was observed in Jurkat, Karpas-299, and K-562 upon TYK2 knockdown. This could have resulted from technical issues from stripping and reprobing (see Materials and Methods) or actual upregulation due to possible compensatory mechanisms following TYK2 depletion.

FIGURE 3. Selective knockdown of TYK2 mRNA by ASO-1.
TYK2 mRNA levels, normalized against GAPDH, of different cell lines (Jurkat, K-562, Karpas-299, MOLT-4, A-431, and HDLM-2) after incubation with 1 μM ASO-1 for 72 h. n = 2, from two independent experiments. mRNA levels of JAK1/2/3 were largely not affected.
Western blot of the cell lysate to determine the potential inhibition of pathway stimulation by ASO-1.

Type I IFNs (IFN-α/IFN-β) signal through binding to the IFN-α/β receptor (IFNAR), which is mediated by TYK2 and JAK1, and eventually lead to the activation of several STAT members, including STAT1/3 (44–46). Following ASO-1 treatment for 3 d, Jurkat and Karpas-299 cells were stimulated with 30 ng/ml IFN-α for 30 min. Western blot of cell lysate showed that STAT1 and STAT3 phosphorylation within both cell lines was inhibited by ASO-1 in a dose-dependent manner (Fig. 4), whereas the total amounts of nonphosphorylated STAT1 and STAT3 remained unchanged. Our results indicated the dependence of type I IFN signaling on TYK2 protein. These are consistent with previous findings from human TYK2 deficiency (9) and TYK2-knockout mice (47, 48), albeit in the mice knockouts, TYK2 protein was found to play a more-limited role in type I IFN.

IL-12 signaling occurs through its binding to the IL-12R, a heterodimeric complex consisting of IL-12Rβ1 and IL-12Rβ2 (49). Upon IL-12 binding to the receptor, the bound TYK2 and JAK2 are activated and phosphorylate STAT1/3/4 (50, 51), thereby leading to downstream gene regulation events, among which include IFN-γ production. After ASO treatment for 3 d, NK-S1 cells were stimulated with 20 ng/ml IL-12 for 1 h. Western blot of the cell lysate showed that phosphorylated STAT4 levels following IL-12 stimulation were reduced in cells that were pretreated with ASO-1, as compared with the controls (Fig. 5). Effects of TYK2 knockdown were also evident from IFN-γ quantification through ELISA measurement, wherein IFN-γ secretion (stimulated over a duration of 12 h with 20 ng/ml IL-12) was drastically reduced in NK-S1 cells pretreated with ASO-1, as compared with nontreated controls and cells pretreated with ASO-ctrl (Supplemental Fig. 2). Our results pointed to the essential role of TYK2 protein in mediating IL-12 signal transduction (9, 47, 48, 52), as has been reported previously from studies in humans (9, 52) and mice (47, 48).

IL-10 signaling occurs through a heterotetrameric receptor complex comprising two each of IL-10R1 and IL-10R2 (53), which is mediated by TYK2 and JAK1, subsequently leading to STAT3 phosphorylation (54). Jurkat and K-562 cells were stimulated with 100 ng/ml IL-10 for 1 h following pre-treatment with ASO-1 for 3 d. Western blot of the cell lysate showed that STAT3 phosphorylation was inhibited in a dose-dependent manner (Fig. 6), in agreement with TYK2 protein depletion, whereas normal STAT3 protein levels were not affected. Our results suggested at least a similar level of involvement from TYK2 in IL-10 signaling as compared with JAK1, which contrast with results from previous studies reporting a dominant role of JAK1 over TYK2 in IL-10 signal transduction (48, 55, 56).

IL-6 signaling is mediated through its receptor complex (57), which is composed of gp130 and IL-6Rα. Upon IL-6 binding, the associated JAK1, JAK2, and TYK2 are activated, thereby leading to phosphorylation of STAT3 protein (9, 10). In this study, we

FIGURE 4. Dose-dependent inhibition of TYK2-mediated IFN-α signaling by ASO-1.
(A) Western blot showing the protein levels of TYK2, STAT1/3, and phosphorylated STAT1/3 in Jurkat cell after IFN-α stimulation for 30 min following incubation with varying doses of ASO-1 for 72 h. (B) Western blot showing the protein levels of STAT1 and phosphorylated STAT1 in Karpas-299 cell, after IFN-α stimulation for 30 min following incubation with varying doses of ASO-1 for 72 h. Experiment was performed on two independent cell lines (n = 2).

FIGURE 5. Inhibition of TYK2-mediated IL-12 signaling by ASO-1.
Western blot showing the protein levels of phosphorylated STAT4 in NK-S1 cell, with or without IL-12 stimulation for 1 h following incubation with 10 μM ASO-1 or the control oligo ASO-ctrl for 72 h. n = 2, from two independent experiments.
measured the levels of phosphorylated STAT3 in A-431 cells after IL-6 (100 ng/ml) stimulation for 1 h following pretreatment with ASO-1 for 3 d. In this case, TYK2 depletion with ASO-1 did not lead to any visible changes on both phosphorylated and normal STAT3 (Supplemental Fig. 3), even at the highest dose tested (2 μM), suggesting minimal impact of TYK2 knockdown on IL-6 signaling through STAT3. Our results were consistent with multiple reports (48, 52, 55, 56, 58) showing a dominant role of JAK1 over JAK2 and TYK2 in mediating IL-6 signal transduction.

We further measured the cytotoxicity of the ASOs through their activation of cellular caspase 3/7 (59, 60) (Supplemental Fig. 4A), which was shown to be a good predictor of their in vivo toxicity. ASO-1 exhibited an excellent safety profile, eliciting a low-caspase activation close to basal level in HeLa adenocarcinoma cells even at a high dose of 1 μM with transfection. In contrast, ASO-3 and ASO-4 led to high relative caspase activation levels (up to ~6–10 times that of untreated control) at the same or lower dose (100 nM) with transfection. It has been reported that cytotoxicity of PS-ASO gapmers could be reduced by chemical modifications (60), specifically through incorporation of 2′-O-methyl (2′-OMe) at gap position 2 (i.e., position 5 of 3′-10-3 gapmers). Accordingly, corresponding modifications to ASO-1, ASO-3, and ASO-4 reduced caspase activation in HeLa cells back to basal levels (Supplemental Fig. 4A) while still retaining their TYK2 mRNA knockdown activity (Supplemental Fig. 4B).

**DISCUSSION**

JAK proteins, acting as critical signaling mediators of many proinflammatory cytokines, represent attractive drug targets for the treatment of autoimmune and inflammatory diseases. Mounting evidence from human genetics studies and clinical findings of several drug agents against TYK2-dependent cytokine signaling pathways (e.g., IFN-α and IL-12/IL-23), combined with the less-severe phenotype of TYK2-knockouts as compared with the other JAK paralogs, indicate TYK2 as an excellent target for the treatment of autoimmune diseases with an optimal balance between efficacy and safety. Additionally, high TYK2 expression has been linked to severe COVID-19 from a recent genome-wide association study of critically ill COVID-19 patients (32), pointing to its selective targeting as a potential treatment for these severe cases. Among small-molecule inhibitors of TYK2 that have been advanced to clinical testing, nearly all were designed to block or compete with ATP binding to the catalytic domain, also known as the Janus Homology 1 (JH1) domain, where selectivity is especially challenging to achieve, given the high homology among the JAK paralogs. A small-molecule inhibitor using an alternative approach of allosteric inhibition through selective binding to the pseudokinase (JH2) domain has shown a remarkable selectivity for TYK2 against all other JAK paralogs, and early clinical results of the inhibitor against PSO (29) look promising. Despite its high selectivity for TYK2, the inhibitor still binds to JAK1 (JH2 domain) and BMPR2 kinase with high affinity (27). Longer-term efficacy and safety of this and other TYK2 inhibitors remain to be determined, and additional TYK2-targeting agents employing other modes of action will build on the drug development toolbox to address the range of autoimmune diseases. With the ongoing pandemic around the world, effective treatment options for severe COVID-19 are urgently needed.

In this study, we have identified potent ASO compounds against TYK2 mRNA. In contrast to small-molecule inhibitors that act through direct binding to the protein target, ASOs operate on the basis of hybridization to the RNA target through complementary base pairing and hence achieve selectivity from a sequence standpoint. In the case of TYK2 ASO gapmers, RNase H are recruited upon ASO hybridization to the sequence target, thereby resulting in specific cleavage and subsequent degradation of TYK2 mRNA and eventually depletion of TYK2 protein. Fully PS-modified LNA gapmers were used in the design of the TYK2 ASOs, which exhibit IC_{50} values matching those of recent ASO candidates with a similar chemical configuration that have been advanced to clinical testing. Such fully chemically modified ASOs are resistant against endo- and exonucleases and remain stable in cells and in vivo for days, even weeks (34, 61). We have also shown that ASO-mediated TYK2 mRNA knockdown can be sustained for at least 4 d posttreatment. Sequence alignments of ASO-1–5 against the human genome transcript database found
no off-target binding sites with significant overlap for the majority of these sequences, hence supporting their specificity for TYK2 mRNA. Experimentally, we have demonstrated their selectivity against the other JAK paralogs, as the mRNA and protein levels of JAK1/2/3 were not reduced by TYK2 ASO treatment.

Thanks to their low molecular mass as well as chemical design, most small-molecule therapeutics typically exhibit excellent cellular uptake profile across all cell types. ASOs, in contrast, display a drastically distinct cellular uptake and pharmacokinetics (PK) profile (61). The differential knockdown efficacies of ASO-1 in various cell lines (IC50 values ranging from ~13 to 155 nM) were consistent with previous observation of variable ASO uptake efficiency across different cell lines (62), even among those originating from the same tissue type. Generally, this would be construed as a disadvantage, as one would normally hope for maximal cellular uptake of a drug compound. However, there could be circumstances under which selective uptake by cellular subsets is desired or cellular uptake into certain cell types is to be avoided. In these cases, selective distribution and uptake of ASO into different cellular subtypes could work to our advantage.

To achieve targeted delivery of ASOs in vivo, intensive efforts have been driven toward direct chemical conjugation of ASO with cell type- and organ-selective ligands (63). Tremendous success has been observed for their targeted delivery to liver hepatocytes upon conjugation with multiple copies of the simple carbohydrate N-acetylgalactosamine (64) (GalNAc). Conjugation of TYK2 ASO with ligands that are specific for immune cell/tissue subtypes of interest thus holds great promise for its targeted delivery to address particular autoimmune diseases. In principle, ligand conjugation can be applied onto small molecules for targeted delivery as well but with certain caveats. First, structure of a small molecule has already been chemically optimized, hence complicating the choices for conjugation chemistry and site of conjugation, and this has to be considered on a case-by-case basis. Compare this against ASO, which is modular by nature and thus would allow the incorporation of diverse chemical functionalities more readily across different ASO sequences. Second, as small molecules are optimized for PK and pharmacodynamics, they might not be compatible with the chemical modification and conjugation method requirements of the ligand. Furthermore, conjugation with ligand could tremendously alter the PK/pharmacodynamics profile of the original small molecule.

Beyond TYK2 mRNA and protein level measurements, we further examined the functional effects of ASO-mediated TYK2 knockdown on various TYK2-associated cytokine signaling pathways (i.e., IFN-α, IL-12, IL-10, and IL-6). In agreement with TYK2 protein depletion by ASO-mediated mRNA degradation, we observed dose-dependent reduction of IFN-α signaling with TYK2 ASO treatment, as measured by pSTAT1/3 levels. Abs against IFN-α and IFNAR have been evaluated for the treatment of SLE, with the latter showing clinical efficacy in a phase III trial (21). Our results were consistent with the essential role of TYK2 in IFN-α signaling, supporting further development of the TYK2 ASOs toward treatment/application on autoimmune diseases such as SLE in which elevated IFN-α signaling was identified as a major driver of pathology (65). On a similar note, we showed that ASO-mediated TYK2 knockdown led to reduction of IL-12 signaling as measured by pSTAT4 levels. This observation was consistent with TYK2 being an important signaling mediator of the IL-12/IL-23 pathway, for which an mAb against the common p40 subunit of IL-12/IL-23 was approved for the treatment of PSO, psoriatic arthritis, Crohn’s disease, and ulcerative colitis (66). We have also shown that IL-10 signaling was inhibited upon TYK2 depletion through ASO treatment, as measured by pSTAT3 levels. Unlike IFN-α and IL-12/IL-23, IL-10 is generally regarded as an anti-inflammatory cytokine (3), although its overproduction has been raised as a predisposing factor for SLE (67). In this case, our results showed that TYK2 exerts an essential role as signaling mediator for IL-10, which contrasts with previous reports on the dominant role of JAK1 over TYK2 in IL-10 signal transduction (48, 55, 56). In contrast, TYK2 depletion showed no effects on IL-6 signaling through pSTAT3, suggesting a limited role for TYK2 in signal transduction of the IL-6 pathway. This was consistent with multiple reports of the dominant role of JAK1 in mediating IL-6 signal transduction (48, 52, 55, 56, 58). We further measured the cytotoxicity of the TYK2 ASOs through cellular caspase 3/7 activation (59, 60), which was shown to be a good predictor of their in vivo toxicity. ASO-1 exhibited an excellent safety profile, whereas cytotoxicity of the other ASOs could be largely eliminated through judicious modifications (60).

The high knockdown efficacy and specificity of the TYK2 ASOs, coupled with their ease of application, make them excellent tool compounds for further investigations on functional roles of TYK2 in immunological settings/autoimmune diseases. Aside from autoimmune diseases, TYK2 was also implicated in the pathology of various cancers, including T cell acute lymphoblastic leukemia (68), anaplastic large cell lymphoma (69), and nerve sheath tumors (70). The TYK2 ASOs presented in this work could be applied to further understand the involvement of TYK2 in the disease pathology of these cancers and serve as a basis for therapeutic development.

In conclusion, we have identified a series of potent TYK2 ASOs that are selective against all other JAK paralogs. The ASOs induce RNase H-mediated RNA degradation upon their binding of TYK2 mRNA, thereby achieving selectivity from the sequence space. This is completely different from small-molecule JAK inhibitors that act by direct binding to the catalytic/allosteric site of TYK2 protein. ASO-mediated knockdown of TYK2 was examined both at the mRNA and protein levels as well as functional measurement of various cytokine signaling pathways that are associated with TYK2. In particular, we have shown that ASO-mediated depletion of TYK2 protein led to reduced activation of IFN-α and IL-12 pathways, for which aberrant signaling has been implicated in the pathogenesis of various autoimmune diseases. These results established the TYK2 ASOs as investigational tool compound and potential therapeutic agent for the treatment of autoimmune diseases and severe COVID-19.
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REFERENCES


Supplemental Figure 1. (A) Western blot showing the proteins levels of TYK2, JAK1, JAK2, and JAK3 in Jurkat cell following incubation with 1 µM of ASO-1 for 24 h, 48 h, and 72 h. (B) mRNA levels, normalized against GAPDH, of TYK2, JAK1, JAK2, and JAK3, in Jurkat cell after incubation with 1 µM of ASO-1–5 for 72 h.
Supplemental Figure 2. IFN-γ secretion by NK-S1 cells, after baseline subtraction of the respective conditions without IL-12 stimulation, as a percentage of untreated control (UTC) without ASO treatment, as determined at OD_{450} from ELISA measurement taken following IL-12 stimulation for 12 h following incubation with 10 µM of ASO-1 or the control oligo ASO-ctrl for 72 h.
Supplemental Figure 3. Western blot showing that the protein levels of STAT3 and phosphorylated STAT3 in A-431 cell line following IL-6 stimulation for 1 h were not affected by pre-treatment with ASO-1.
Supplemental Figure 4. (A) Relative caspase 3/7 activation in HeLa cell induced by TYK2 ASOs and their corresponding counterparts with 2’-OMe at gap position 2. ETC, empty transfection control. (B) TYK2 mRNA levels, normalized against GAPDH, of HDLM-2 cell after incubation with 1 μM of TYK2 ASOs and their corresponding counterparts with 2’-OMe at gap position 2 for 2 days. UTC, untreated control.