Effects of Combined Administration of Imatinib and Sorafenib in a Murine Model of Liver Fibrosis

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Abstract: Liver fibrosis is defined as excessive extracellular matrix deposition in the hepatic parenchyma as a consequence of complex interactions among matrix-producing hepatic stellate cells (HSCs) and liver-resident and infiltrating cells. In addition to the liver, the process of fibrosis may represent end-stage disease of several diseases including kidneys, lungs, spleens, heart, muscles and at certain extent, the central nervous system and the peripheral nerves. To date, antifibrotic treatment of fibrosis represents an unconquered area for drug development. The aim of the present study was to test the efficacy of a new drug combination for the treatment of hepatic fibrosis in order to provide a proof-of-concept for the use of therapeutic agents in clinical practice. For this purpose, we have studied the effects of the PDGF inhibitor imatinib and the angiogenesis inhibitor sorafenib, administered alone or in combination, in reducing the progression of the fibrogenetic process in a pre-clinical model of liver damage induced in mice by repeated administration of Concanavalin A (ConA), resembling long-term autoimmune hepatitis. Our results suggest that treatments with imatinib and sorafenib can modulate potently and, in a superimposable fashion, the fibrinogenic process when administered alone. However, and in agreement with the computational data presently generated, they only exert partial overlapping antifibrotic effects in modulating the main pathways involved in the process of liver fibrosis, without significant additive or synergist effects, when administered in combination.

Keywords: liver fibrosis; imatinib; sorafenib; traumatic spinal cord injury

1. Introduction

Fibrosis represents an intrinsic response to chronic injury, maintaining organ integrity when extensive necrosis or apoptosis occurs. With protracted damage, fibrosis may progress toward excessive scarring and organ failure. Fibrosis occurs in most human organs including the liver, lung, heart, and kidney, and is crucial for the progression of most chronic diseases [1]. In addition, fibrotic scar formation through fibroblasts and pericytes may also contribute to abnormal functioning and lack of recovery of the blood-brain barrier (BBB) during acute and chronic pathologies of the central nervous system such as traumatic spinal cord injury and multiple sclerosis [2,3]. Therefore, fibrosis may represent a common hallmark with common and subtle pathogenetic differences among different organs that jeopardizes organ functionality and often represents an end-stage disease of the affected organ.
Importantly, fibrosis is no longer considered static, but it is instead considered to be the result of a continuous remodeling process. Aberrant activity of transforming growth factor β (TGF-β) or members of the platelet-derived growth factor (PDGF) family are the most prominent drivers of cellular activation and trans-differentiation of hepatic stellate cells (HSCs) into myofibroblasts [4–6]. To date, antifibrotic treatment of fibrosis represents an unconquered area for drug development with enormous potential. Animal models are still the gold standard for basic liver fibrosis research, especially due to the complex interactions among several cell types (hepatocytes, immune cells, and HSCs) during the fibrogenetic process, which is challenging to mimic in vitro [7]. As a consequence, various surgical, genetic, toxic, and nutritional models have been widely applied and serve as models for the different types of fibrosis observed in humans [8].

The better understanding of the pathogenetic processes implicated in fibrosis has helped development of pirfenidone, which is an orally available pyridinone derivative that inhibits collagen formation primarily, but not exclusively, through inhibition of two molecules implicated in fibrosis, such as TGF-beta and PDGF. Pirfenidone has been approved for the treatment of IPF and is being studied for the treatment of other forms of fibrosis [9–11]. However, response to pirfenidone is suboptimal for most patients and may be associated with some side effects [12]. Therefore, development of new drugs that, either alone or in combination with pirfenidone, may tackle the fibrinogenic process is urgently needed.

Along this line of research, we have studied the ex vivo and in vivo effects of two compounds that are currently used for the treatment of several forms of cancer—the anti-PDGF, imatinib, and the VEGF inhibitor, sorafenib—in counteracting biomolecular fibrinogenic pathways and histological appearance of fibrosis in a mouse model of liver fibrosis induced by repeated injections of ConA.

From the histological and pathogenetic points of view, liver fibrosis is defined as excessive extracellular matrix deposition, and is based on complex interactions between matrix-producing HSCs and a variety of liver-resident and infiltrating cells [13–16]. Liver cirrhosis represents the major risk factor for the development of hepatocellular carcinoma with an important clinical impact on the management and prognosis of patients with primary liver cancer [17].

The aim of the present study was to test the efficacy of a new drug combination for the treatment of hepatic fibrosis in order to provide a proof-of-concept for the use of therapeutic agents in clinical practice. For this purpose, we have studied the effectiveness of the PDGF-inhibitor, imatinib, and of the angiogenesis inhibitor, sorafenib, administered alone or in combination, in reducing the progression of the fibrogenetic process in a pre-clinical model of liver damage induced in mice by repeated administration of Concanavalin A (ConA).

2. Results

2.1. Animal Study

2.1.1. Effects of ConA Injection

The experimental design is shown in Figure 1 and Table 1. Administration of ConA (10 mg/kg) was associated with a mortality of approximately 35%, with a peak incidence between the second and third week (data not shown). Transaminases were measured 24 h after each ConA administration. All animals included in the study showed significant increases in transaminases levels during the period of ConA administration. As shown in Supplementary Figure S1, a significant increase in GPT levels was observed already after the first ConA administration, and reached a peak after the second administration (Supplementary Figure S1). After the last dose of ConA, the levels of transaminase were not significantly higher than those observed in the sham-unchallenged mice, suggesting the progressive exhaustion of the liver parenchyma. After the last ConA administration, the animals were randomized to obtain a homogeneous number of animals for each experimental group.
2.1.2. Effects of Imatinib and Sorafenib on the Expression of Alpha-SMA

ACTA2 (commonly referred to as alpha-smooth muscle actin or α-SMA) is often used as a marker of myofibroblast formation, and it plays an important role in fibrogenesis [18]. Myofibroblasts are metabolically and morphologically distinctive fibroblasts expressing alpha-SMA, and their activation plays a key role in development of the fibrotic response. In an activated state, myofibroblasts stop to proliferate and start to synthesize large amounts of extracellular component proteins. Expression analysis of alpha-SMA showed an increase, although not significant (~37%), in the group of vehicle-treated animals, compared to sham mice (Figure 2A). Treatments with imatinib and sorafenib were associated with a reduction in alpha-SMA levels of 51% and 34%, respectively, compared to the vehicle (statistical significance was not reached) (Figure 2A). No additive or synergistic effects were observed between imatinib and sorafenib as, in the group of animals treated with both drugs, alpha-SMA levels were found to be reduced by 40% compared to the vehicle (Figure 2A).
2.1.3. Effects of Imatinib and Sorafenib on the Expression of Collagens

As shown in Figure 2B, a significant increase in COL1A1 expression was found in the vehicle groups as compared to the sham group (p < 0.01). Treatments with imatinib and sorafenib resulted in a reduction in COL1A1 levels, compared to the vehicle (p < 0.05 and p < 0.01, respectively). A significant reduction (p < 0.05) in COL1A1 levels was also observed in the group treated with the combination of imatinib and sorafenib (Figure 2B). The analysis of COL1A2 expression showed an increase of 46% in the vehicle group compared to sham animals (p < 0.01) (Figure 2C). Treatments with imatinib and sorafenib resulted in a reduction in COL1A2 levels of 38% and 44%, respectively, compared to the vehicle (p < 0.05 and p < 0.01, respectively) (Figure 2C). The imatinib–sorafenib combination was associated with a 37% reduction in COL1A2 levels compared to vehicle group (p < 0.05) (Figure 2C). A similar modulation in expression was found for COL3A1, as the administration of imatinib, sorafenib, and the imatinib–sorafenib combination was associated with a marked (p < 0.001) reduction in COL1A3, as compared to the vehicle (Figure 2D).

**Figure 2.** Effects of imatinib and sorafenib on the expression of ACTA2 and collagens. Mice were challenged with Concanavalin A for four weeks and then treated with either imatinib, sorafenib, a combination of imatinib and sorafenib, or vehicle for three weeks. At the end of the treatment period, mice were euthanized and their livers were collected for the determination of alpha-SMA (ACTA2) (A), COL1A1 (B), COL1A2 (C), and COL3A1 (D) via real-time PCR. * p < 0.05; ** p < 0.01; *** p < 0.001.
2.1.4. Effects of Imatinib and Sorafenib on the Expression of Transforming Growth Factor Beta (TGFβ)1 and TGFβ2 and of Platelet-Derived Growth Factor (PDGF)

The transforming growth factor-beta (TGF-β) family plays relevant roles in the regulation of different cellular processes that are essential for tissue and organ homeostasis [14]. In chronic liver diseases, TGF-β signaling participates in different stages of disease progression, from acute liver injury toward fibrosis, cirrhosis, and cancer [4–7]. When a chronic injury takes place, mobilization of lymphocytes and other inflammatory cells occurs, thus setting the stage for persistence of an inflammatory response. Macrophages produce profibrotic mediators—among them, TGF-β, which is responsible for activation/transdifferentiation of quiescent hepatic stellate cells (HSC) to a myofibroblast (MFB) phenotype.

In the present experimental study, we observed a significant ($p < 0.001$) increase in TGFβ1 expression in the vehicle group, as compared to sham mice (Figure 3A). The administration of imatinib, sorafenib, and the imatinib–sorafenib combination was associated to a marked reduction in TGFβ1, as compared to the vehicle ($p < 0.05$ for imatinib, and $p < 0.01$ for sorafenib and the combination treatment) (Figure 3A).

The analysis of TGFβ2 expression levels showed an increase of 13% in the vehicle group compared to sham animals ($p > 0.05$) (Figure 3B). Treatments with imatinib and sorafenib resulted in a reduction in TGFβ2 levels by 21% and 35%, respectively, compared to the vehicle ($p > 0.05$ and $p < 0.05$, respectively) (Figure 3A). The association between imatinib and sorafenib did not modulate TGFβ2 levels compared to the vehicle group (Figure 3B).

As shown in Figure 3C, there was a significant ($p < 0.001$) increase in PDGFB expression in the vehicle group, as compared to sham mice (Figure 3C). The administration of imatinib, sorafenib, and the imatinib–sorafenib was associated with a marked reduction in TGFβ1, as compared to the vehicle ($p < 0.01$ for imatinib and the combination treatment, and $p < 0.001$ for sorafenib) (Figure 3C).

2.1.5. Effects of Imatinib and Sorafenib on the Expression of Interleukin 6 (IL-6)

Interleukin-6 (IL-6) is a protein synthesized by fibroblasts, monocytes, macrophages, T cells, and endothelial cells. IL-6 synthesis and secretion is induced during inflammatory conditions such as upon stimulation of Toll-like receptor (TLR)-4 by lipopolysaccharide or upon stimulation of cells by IL-1 or tumor necrosis factor (TNF)-α. Additionally, IL-6 has profibrotic properties, and it is also involved in liver regeneration after hepatectomy [19]. IL-6 can directly promote the transition of hepatic stellate cells (HSCs) toward myofibroblast-like cells, and this effect is mediated by the activation of the MAPK and JAK/STAT signaling pathways. Inhibition of either MAPK or JAK/STAT signaling pathways dampens HSC stimulation [20]. The vehicle group showed an IL-6 increase of 52% compared to the sham group ($p < 0.05$) (Figure 3B). Treatment with imatinib reduced IL-6 levels by 75% ($p < 0.001$), while sorafenib and the imatinib–sorafenib combination resulted in a mild increase of this cytokine level in the liver tissue (15%, $p > 0.05$, and 47%, $p < 0.05$, respectively) compared to the vehicle (Figure 3A).
2.1.6. Effects of Imatinib and Sorafenib on the Expression of Tissue Inhibitor Metalloproteinases (TIMP)1, TIMP2, Matrix Metallopeptidase (MMP)2, and MMP9

TIMPs are the primary inhibitors of metalloproteinases (MMPs), a group of specific proteins which play an important role in tissue remodeling and deposition of extracellular matrix during the fibrogenesis process [4,14]. Although HSCs clearly play a role in matrix protein synthesis, they are also able to regulate matrix degradation. In the early phases of activation, HSCs release MMPs with the ability to degrade normal liver matrix. When HSCs are fully activated, there is a net downregulation of matrix degradation, reflected by increased HSC synthesis and release of TIMPs 1 and 2. Kupffer cells also are capable of releasing TIMPs.

In this experimental study, the vehicle group showed a TIMP1 increase of 51% compared to the sham group (p < 0.001) (Figure 4). Treatments with imatinib and sorafenib reduced TIMP1 levels by 26% (p < 0.05) and 30% (p < 0.05), respectively, while in the group treated with the combination of the two aforementioned drugs, only a non-significant reduction of 20% was observed (Figure 4A). Similar data were obtained for TIMP2, whose expression was significantly increased in the vehicle group,
as compared to the sham group (Figure 4B). Imatinib and sorafenib administration were associated to a
significant reduction in TIMP2 expression ($p < 0.01$ and $p < 0.05$, respectively). However, the statistical
significance was not reached for the imatinib–sorafenib combination, which entailed a moderate
reduction in TIMP2 expression as compared to the vehicle (Figure 4B).

Analysis of the expression of MMP2 and MMP9 revealed a significant upregulation of both
transcripts in the vehicle group ($p < 0.05$ and $p < 0.001$, respectively) (Figure 4C,D). Sorafenib was
associated to a significant reduction in MMP2 expression ($p < 0.05$) (Figure 4C), as compared to the
vehicle, while only a trend of reduction was observed in the imatinib and in the imatinib–sorafenib
combination groups (Figure 4C). On the other hand, all the treatment groups showed a significant
reduction in MMP9 expression, as compared to the vehicle group ($p < 0.01$ for imatinib, and $p < 0.05$
for sorafenib and the imatinib–sorafenib association) (Figure 4D).

![Figure 4](image-url)

**Figure 4.** Effects of imatinib and sorafenib on the expression of TIMP1, TIMP2, MMP2, and MMP9.
Mice were challenged with Concanavalin A for 4 weeks and then treated with either imatinib, sorafenib,
a combination of imatinib and sorafenib, or vehicle for three weeks. At the end of the treatment period,
mice were euthanized and their livers were collected for the determination of TIMP1 (A), TIMP2 (B),
MMP2 (C), and MMP9 (D) via real-time PCR. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$;
2.1.7. Histopathological Analysis

Representative histological pictures of the liver from the experimental groups are shown in Figure 5A–E. No significant modulation in the inflammatory and necrosis index was observed in treated groups compared to the vehicle group (Figure 5F–H). Imatinib, sorafenib, and the imatinib–sorafenib combination reduced the histological evidence of liver fibrosis compared to the vehicle group using METAVIR scoring of H&E-stained liver sections (Figure 5).

![Figure 5](image)

**Figure 5.** Liver histology. Representative H&E microphotographs of the liver from the (A) sham healthy group; (B) vehicle-treated group; (C) imatinib-treated group; (D) sorafenib-treated group; (E) imatinib–sorafenib-treated group. Scores for inflammation (F), necrosis (G), and fibrosis (H) are shown as mean ± s.d.. **p < 0.01; ***p < 0.001.

2.2. In Silico Analysis

In order to determine the molecular basis for the observed lack of synergistic/additive effects of imatinib and sorafenib, we calculated the “consensus perturbation signature” for imatinib and sorafenib and determined the cellular response to these drugs on pro- and anti-fibrogenetic genes. The genes selected included 44 genes, such as IL-6 and TGF-beta, and their cognate receptors/co-receptors, genes encoding for components of the extracellular matrix (collagens, fibronectin, tenascin C, and alpha-SMA), matrix metalloproteinases, and tissue inhibitors of metalloproteinases. Figure 6 shows the gene network constructed with the selected genes, which are differently modulated by imatinib and sorafenib, both in terms of degree of regulation and, sometimes, direction of modulation (Figure 6).
In our study, we have investigated the liver expression levels of alpha-SMA (ACTA2) and of fibrillar collagen COL1A1, COL1A2, and COL3A1; of the pro-fibrogenetic cytokines TGFβ1, TGFβ2, PDGFB and IL-6; as well as, the levels of the metalloproteinase inhibitors TIMP1 and TIMP2; and of the matrix metalloproteinases, MMP2 and MMP9 in a model of liver fibrosis induced by repetitive treatment of mice with ConA. This model stems from our previous experience with the ability of one single administration of ConA to induce autoimmune hepatitis (AIH) in mice within 18–24 hours from injection. AIH is an immune-mediated inflammatory disorder of the liver that occurs at all ages, with women being more affected. Although most of the AIH patients respond to the standard-of-care (SOC) treatment consisting of prednisone and azathioprine, a significant percentage of them show partial or poor responses and can progress to liver fibrosis, and eventually cirrhosis [21]. The observation of the progression of some AIH patients to liver fibrosis over progression of the disease prompted us to evaluate the effects of a prolonged challenge with hepatitis-inducing doses of ConA in mice. In fact, the prolonged challenge with ConA resulted in development of mild fibrosis in the group of so-challenged mice that were accompanied by clear activation of profibrogenic pathways, including TGF-b and IL-6.

Several independent studies have already shown that imatinib, via its inhibitory effect on PDGFR, and sorafenib, via its double inhibitory action on VEGFR/PDGFR and RAF/MEK/ERK pathways, play an important anti-fibrotic role both in vitro and in vivo [22–30]. In the present study, we aimed to test the effects of these drugs in a different model of liver fibrosis which more closely resembles the liver degeneration and fibrotic processes that occur with immune-mediated liver disorders such as autoimmune hepatitis, acute viral hepatitis, and drug-induced immune activation [31,32].

In our study, we have investigated the liver expression levels of alpha-SMA (ACTA2) and of fibrillar collagen COL1A1, COL1A2, and COL3A1; of the pro-fibrogenetic cytokines TGFβ1, TGFβ2, PDGFB and IL-6-; as well as, the levels of the metalloproteinase inhibitors TIMP1 and TIMP2; and of the matrix metalloproteinases, MMP2 and MMP9 in a model of liver fibrosis induced by repeated administrations of ConA to mice, resembling the course of AIH in patients poorly responsive to SOC treatment. Our results suggest that imatinib and sorafenib exert only a partial overlapping...
effect in inhibiting the main pathways involved in the process of liver fibrosis. An important aspect regarding the experimental layout is represented by the fact that the treatments were administered to the animals in therapeutic regimen, starting from a week after the fourth and last administration of ConA. This differentiates our analysis from other precedent studies, as this experimental regimen allowed us to study the effect of treatments exclusively on the fibrogenic process, excluding the possible protective effect of drugs against the acute hepatic injury.

It should be noted that only an initial and low-grade fibrosis was observed upon ConA challenge in the vehicle-treated animals. It is likely that longer periods of times, or additional doses of ConA, are necessary to obtain more profound alterations in liver morphology. However, as the fibrogenetic process is a dynamic event which progressively becomes irreversible, it may be useful to study the effects of potential novel drugs when a low/moderate fibrosis is in place, in order to provide proof-of-concept data with a more immediate translational value.

Although both imatinib and sorafenib inhibit the tyrosine kinase activity of PDGFR, the two drugs have different pharmacokinetics and pharmacodynamics, as demonstrated by the inhibitory effect of sorafenib on MAP kinases. Therefore, we aimed to verify the effects of the simultaneous administration of the two drugs on the hepatic fibrogenetic process. No additive or synergistic effects were observed for the two drugs, since the degree of modulation of the aforementioned molecules was not significantly different in the group of animals treated with imatinib + sorafenib compared to the groups treated with the two drugs alone. This data can be explained in light of the bioinformatic analysis, which showed different and sometimes, opposite, effects of imatinib and sorafenib on the modulation of the expression of known fibrogenetic genes. The use of whole-genome expression data has been largely used to identify pathogenic pathways and therapeutic targets [33–41] for several disorders, including autoimmune diseases [42,43] and cancer [44–50].

However, gain-of-function and gene knockdown studies on specific liver subpopulations—in particular, HSCs and portal fibrobasts—would be needed to deepen the knowledge on the processes underlying liver fibrosis and the real in vivo effects of such pharmacological treatments.

In conclusion, our study confirms and reinforces the data previously reported in the literature on the possible use of tyrosine kinase inhibitors as antifibrotic agents. However, the model used does not allow us to verify whether or not these drugs have the possibility of reverting the fibrotic process or, rather, only slowing it down/preventing it. In fact, to solve a condition of pre-existing fibrosis, it would be necessary to activate the digestion of the extracellular matrix by acting on the interstitial proteases and collagenases and, at the same time, promoting hepatocyte regeneration for the maintenance of a functional hepatic micro/macrosopic structure.

4. Materials and Methods

4.1. Animal Study

4.1.1. Induction of Liver Fibrosis and Experimental Treatments

Female Balb/c mice of 8–10 weeks, used for the experimental study, were purchased from Harlan Laboratories (San Pietro al Natisone, Udine, Italy). The animals were kept in standard laboratory conditions with free access to food and water, and before starting the study, they were allowed to stall for a week to allow them acclimatization to the new environment. The animal protection complies with the 86/609/EEC directive, reaffirmed by the Italian Legislation D.L. No 116 of 27 January 1992. The experimental study was approved by the Institutional Animal Care and Use Committee (IACUC) of University of Catania.

The experimental design is shown in Figure 1 and Table 1. The protocol treatment is summarized in Table 1. The control group consisted of animals that received the Concanavalin A (ConA) and were treated with the drug vehicle (negative control group). A group of animals received only the ConA vehicle representing the group of healthy mice (sham). During all the experimental procedures, it was
the care of the researcher to act with respect for the animals, ensuring them the least possible suffering and avoiding unnecessary pain. Administration of ConA was performed intravenously via tail vein injection at a dose of 10 mg/kg once a week for four consecutive weeks. 24 h after each ConA injection, transaminase levels (GPT) in serum samples were evaluated using a Reflotron® analyzer. One week after the fourth administration of ConA, the treatment protocol started. All treatments were performed five times a week for three consecutive weeks.

4.1.2. Ex Vivo Analysis

One week after the end of the experimental treatment, all mice were sacrificed by CO2 inhalation in a sealed chamber according to an international standard procedure and blood and liver tissue samples were collected. The right lobe of the liver was fixed in 10% buffered formalin, while the left lobe was placed in TRIzol reagent (Life Technologies Corporation®).

Formalin-fixed paraffin-embedded liver biopsy samples (5 μm thick sections) were stained with hematoxylin and eosin (H&E) to evaluate the degree of liver fibrosis and inflammation/necrosis. The METAVIR score was used to quantify the liver fibrosis severity index. Inflammation was evaluated as follows: 0, no inflammation; 1, periportal inflammation; 2, mild to moderate portal inflammation; 3, severe portal inflammation. Necrosis was evaluated as follows: 0, no necrosis; 1, focal necrosis; 2, interface hepatitis; 3, confluent necrosis.

4.1.3. Real-Time PCR

Total RNA was isolated from samples placed in TRIzol, following the supplier's instructions. Briefly, the samples were centrifuged at 12,000 RCF (relative centrifugal force) after addition of chloroform (500 μL per ml of TRIzol). The aqueous phase was collected and the RNA was precipitated with isopropanol. Finally, the RNA was washed by adding 75% ethanol in diethylpyrocarbonate (DEPC) water. The quantification and purity of the RNA thus extracted was assessed by determining the absorbance at 260/280 nm. Two micrograms of RNA for each sample were retro-transcribed by using the FirstStrand cDNA Synthesis kit (Roche, Monza, Italy), and the cDNA was used for real-time PCR using the FastStart SYBR Green Master kit (Roche, Monza, Italy) [51,52].

The sequences of the primers used were the following: ACTA2 forward: GTCCCAGACATCCGGAGTAA; ACTA2 reverse: TCGGATACCTCAGCCTCAGGA; interleukin-6 (IL-6) forward: TAGTCCCTTCTACCCAATTTCC; IL6 reverse: TTGGTCTTCAGCCTCCTTC; TGFβ1 forward: ATGTACCGGTAGGGGTC; TGFβ1 reverse: GGCCTGCACTGTGCTGTATAG; TGFβ2 forward: TCGACATCGACTGTATGCG; TGFβ2 reverse: CCCTGGTGACTGTAGTAGATG; PDGFB forward: CATTCCGGCTCTTGTGATCT; PDGFB reverse: GTGCTCGGTCATGTACAG; COL1A1 forward: GTGCTCGGTCATGTACAG; COL1A1 reverse: CCACGTCTACCATGGGG; COL2A1 forward: GTACCTCTGCCTTTGAAACA; COL2A1 reverse: CCTTGTACAGAATCTGAGC; TIMP1 forward: CCGCCCGGACTGAAATC; TIMP1 reverse: CGCGCCGTAGAACC; TIMP2 forward: TCGAGCCCGAGAGTTG; TIMP2 reverse: GCCGTGTAGAAGACTC; MMP2 forward: CAATCCGGAGTATG; MMP2 reverse: TTCTGGTGATAGG; MMP9 forward: CGCAGCAGACACATTAAG; MMP9 reverse: CTCGCAGCAACTCAGAG; beta-actin forward: CATATCCTACCTACCTAG; beta-actin reverse: GCCATCTGTCAGGACCC.

The level of gene expression, normalized towards the control, was calculated using the following formula: $2^{-\Delta Ct}$, where $\Delta Ct = (C_t \text{ target gene} - C_t \text{ beta-actin}).$

4.2. In Silico Analysis

The “consensus drug signature” for imatinib and sorafenib was obtained by a meta-analysis of the Library of Integrated Network-based Cellular Signatures (LINCS) L1000 perturbation data [53]. Currently, the L1000 transcriptomic profiles represent >40,000 genetic and small molecule perturbations, profiled across a variable number of cell types. The L1000
data is provided at five levels of the data processing pipeline: raw unprocessed flow cytometry data from Luminex (Level 1); gene expression values per 1000 genes after deconvolution (Level 2); quantile-normalized gene expression profiles of landmark genes and imputed transcripts (Level 3); gene signatures computed using z-scores relative to the control (Level 4); and differential gene expression signatures (Level 5) [54]. For the analysis, the 978 measured landmark genes and the 6489 best imputed genes were used. The Stouffer’s method for the meta-analysis of the z-scores [55] was employed to obtain the consensus drug signature [56]. The Cytoscape software was used to visualize the gene modulation exerted by imatinib and sorafenib in the network constructed with the genes of interest using the GeneMania interaction data.

### 4.3. Statistical Analysis

Statistical analysis was performed using the ANOVA test or Kruskal–Wallis test, followed by either Fisher’s LSD or Dunn’s post-hoc multiple comparison test, based on the results obtained from the Shapiro–Wilk, D'Agostino–Pearson, and Kolmogorov–Smirnov normality tests. The GraphPad Prism (v. 5) software was used for the statistical analysis and the generation of the graphs. A p-value < 0.05 was considered statistically significant.

### 5. Conclusions

To date, the antifibrotic treatment of fibrosis represents an unconquered area for drug development, with enormous potential but also high risks. Preclinical research has yielded numerous targets for antifibrotic agents, some of which have entered early-phase clinical studies, but progress has been hampered due to the relative lack of sensitive and specific biomarkers to measure the progression or reversal of fibrosis. Antifibrotic therapies should also be customized on the basis of disease-specific features and patient genetic characteristics, and multidrug approaches targeting mechanistically distinct components of the fibrogenic pathway could be pursued with the aim of having fewer and less toxic adverse effects. The possibility of delaying or halting the progression of fibrosis will be particularly important for those patients for whom disease-specific treatment is either not available or ineffective to preserve liver function, reducing the complications of cirrhosis and delaying the need for liver transplantation. Our study confirmed the effectiveness of tyrosine kinase inhibitors as antifibrotic agents by modulating the expression of the main molecules involved in the liver fibrosis process. However, we observed no additive or synergistic effects for the two drugs. This observation, despite being counterintuitive, was supported by our in silico analysis, which showed that imatinib and sorafenib often induce diverging modulation on genes involved in the fibrogenetic processes.

### Supplementary Materials

The following are available online, Figure S1: Serum ALT levels during the course of ConA challenge.

### Author Contributions

Conceptualization, A.P., P.F., P.B., F.N., and K.M.; data curation, A.P., R.C., A.B., E.C.A.I., M.C.P., G.G.M., P.F., and K.M.; formal analysis, R.C., A.B., E.C.A.I., and M.C.P.; funding acquisition, P.B.; methodology, M.C.P., G.G.M., and P.F.; project administration, P.B.; writing—original draft, A.P., R.C., and A.B.; writing—review and editing, G.G.M., P.F., F.N., and K.M. All authors have read and agreed to the published version of the manuscript.

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### Conflicts of Interest

The authors declare no conflict of interest.

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**Sample Availability:** Samples of the compounds are available from the authors upon reasonable request.

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