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C o o r d i n a t o r e : P r o f . R o b e r t o A v o l a

DOTT. ANTONIO PESCE

Effects of Imatinib, Sorafenib and CORM-A1 in a pre-clinical model of liver fibrosis

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Tesi di Dottorato

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SUMMARY

Effects of Imatinib, Sorafenib and CORM-A1 in a pre-clinical model of liver fibrosis

Abstract : Liver fibrosis is defined as excessive extracellular matrix deposition and is based on complex interactions between matrix-producing hepatic stellate cells (HSCs) and an abundance of liver-resident and infiltrating cells. Fibrosis represents an intrinsic response to chronic injury,

maintaining organ integrity when extensive necrosis or apoptosis occurs. Following liver injury of any kind, a defined program of molecular changes occurs that is highly orchestrated at the cellular and molecular levels. Animal models are still the gold standard for basic liver fibrosis research, especially due to the complex interaction of several cell types (hepatocytes, immune cells and HSCs) during fibrogenesis, which is challenging to mimic in vitro. As a consequence, various surgical, genetic, toxic and nutritional models are widely applied and serve as models for the different types of fibrosis observed in humans. To date, the antifibrotic treatment of hepatic fibrosis represents an unconquered area for drug development, with enormous potential.

The aim of the present study was to test the efficacy of new drug therapies 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 analyzed and studied the effectiveness of the PDGF-inhibitor Imatinib, of the angiogenesis inhibitor - Sorafenib, administered alone or in combination, and of the carbon monoxide release - CORM-A1, 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.

Introduction

Fibrosis and cirrhosis are both strictly defined pathological entities that were broadly defined by pathologists and hepatologists several decades ago [1,2]. Liver fibrosis is defined as excessive extracellular matrix deposition and is based on complex interactions between matrix-producing hepatic stellate cells (HSCs) and an abundance of liver-resident and infiltrating cells. Fibrosis is an intrinsic response to chronic injury, maintaining organ integrity when extensive necrosis or apoptosis occurs. With protracted damage, fibrosis can progress toward excessive scarring and organ failure, as in liver cirrhosis. Although hepatic fibrosis in humans can be caused by various stimuli (congenital, metabolic, inflammatory, parasitic, vascular, toxins or drugs), the molecular mechanisms underlying fibrosis are basically the same, as shown in *Figure 1* [3]. Following liver injury of any kind, a defined

program of molecular changes occurs that is highly orchestrated at the cellular and molecular levels [4]. Importantly, fibrosis is no longer considered static, but the result of a continuous remodeling process. This process is characterized mainly by cellular activation of hepatic stellate cells (HSCs) which acquire a myofibroblast (MFB) phenotype and are able to express and deposit large quantities of extracellular matrix (ECM) components within the liver [5,6]. If the injury is temporarily, these changes are transient and liver fibrosis may resolve. If the injury is sustained, however, chronic inflammation and accumulation of the ECM persist, leading to progressive substitution of normal liver parenchyma by scar tissue, as shown in *Figure 2*. In the pathogenesis of chronic liver disease, ECM homeostasis is further disturbed by an unbalanced activity of matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs). Experimental studies that were conducted in isolated primary hepatic cells and experimental animal models led to the identification of general pathogenetic mediators-signalling pathways that are involved in the fibrogenic response. Aberrant activity of transforming growth factor β2 (TGF-β2) or members of the platelet-derived growth factor (PDGF) family are the most prominent drivers of cellular activation and trans-differentiation of HSCs into MFBs. To date, antifibrotic treatment of fibrosis represents an unconquered area for drug development, with enormous potential but also high risks.

However, several questions remain unanswered : Can we pharmacologically accelerate fibrosis resolution in humans ? Can a fibrotic liver completely regress to a normal liver ? Does fibrosis reverse similarly in all types of liver diseases ? Although isolated cases of complete fibrosis resolution have been reported, it is conceivable that some degree of fibrosis cannot be removed. Resolution may be limited by ECM cross-linking and a failure of activated HSCs to undergo apoptosis. The research is going in this direction in order to resolve some of these questions.

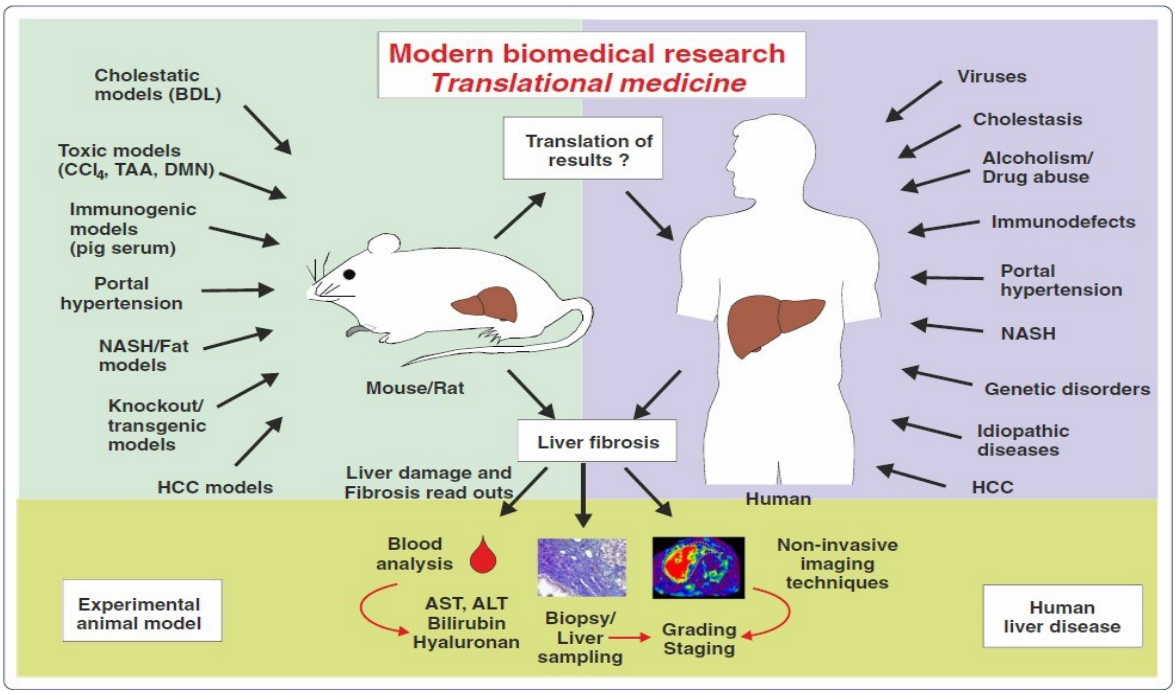
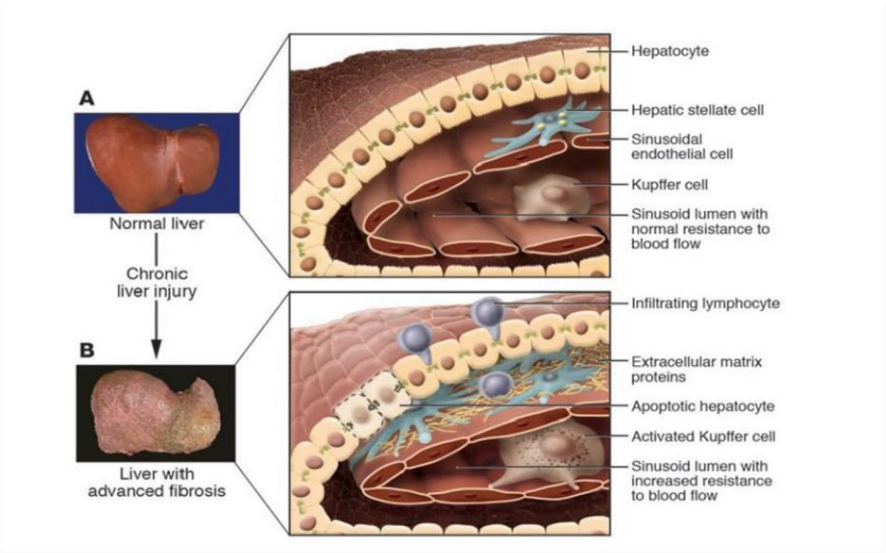


Figure 1. Experimental animal models and human liver diseases involved in hepatic fibrosis. (From *Liedtke C et al.* Fibrogenesis Tissue Repair. 2013)



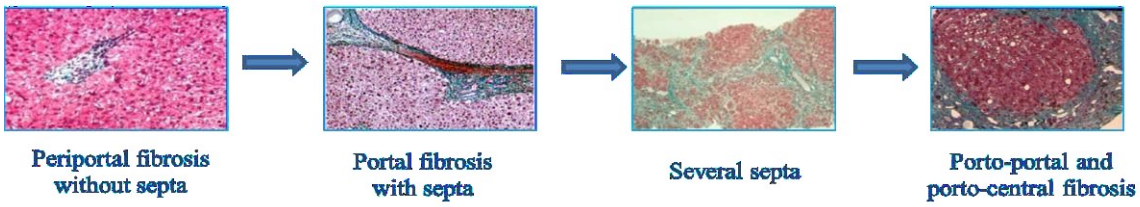


Figure 2. Pathogenesis of liver fibrosis: from the activation of hepatic stellate cells (HSC) to deposition of extracellular matrix (ECM) and formation of fibrotic septa in the porto-portal and porto-central anatomic spaces.

Pre-clinical models of liver fibrosis

As outlined above, animal models are still the gold standard for basic liver fibrosis research, especially due to the complex interaction of several cell types (hepatocytes, immune cells and HSCs) during fibrogenesis, which is challenging to mimic in vitro. As a consequence, various surgical, genetic, toxic and nutritional models are widely applied and serve as models for the different types of fibrosis observed in humans [7]. The *Figure 3* highlights the different mouse models of liver fibrosis in pre-clinical research. The BDL (Bile Duct Ligation) model is a surgical model that induces mainly cholangiocyte proliferation with consecutive formation of peribiliary plexus and portal fibrosis, leading to portal hypertension and cholestatic fibrosis and shunts within four to six weeks [8].The main advantages of this model are technical feasibility, short time to achieve typical disease, reproducibility and high similarity with humans in terms of portal hypertension (*Figure 4*). There are also many experimental models of toxic fibrosis described in scientific literature, for example by carbon tetrachloride (CCl4), thioacetamide (TAA), dimethylnitrosamine (DMN) treatment : they are highly reproducible, fast, resemble properties of human fibrosis and present a good comparability due to abundant reference studies [9-12].

The most commonly used approach to induce toxin-mediated experimental liver fibrosis is the periodic administration of carbon tetrachloride (CCl4) in mice or rats. In mice, typically 0.5 to 2 ml/kg body weight CCl4 (diluted in corn oil) is injected intraperitoneally (i.p.) two to three times per week, resulting in robust and highly reproducible liver fibrosis between 4 and 6 weeks of treatment. Thus, a single CCl4 injection in mice can also be used as an attractive and highly reproducible model of liver regeneration after toxic injury. The first appearance of histological fibrosis and scarring fibres is usually observed after 2 to 3 weeks of CCl4 treatment, depending on the dosage and mouse strains used. Moreover, CCl4-induced liver fibrosis in mice can be completely resolved within several weeks after withdrawal of the toxic treatment. For all the above mentioned reasons, the CCl4 model resembles all important properties of human liver fibrosis, including inflammation, regeneration, fibre formation and potentially fibrosis regression.

Table 1 Overview of mouse models of liver fibrosis[a]

Animal model	Intervention	Advantage	Disadvantage	Type of fibrosis	Reference
Bile duct ligation (BDL)	Surgical	Fast and highly reproducible		Cholestatic fibrosis	[22]
Mdr2$^{-/-}$ mice	Genetic	Well-reproducible	Long latency (3 to 6 months)	Sclerosing cholangitis/ biliary fibrosis	[27]
Dominant-negative *TGFβRII* mice	Genetic	Resembles human disease		Primary biliary cirrhosis (PBC)	[28]
Il-2Rα$^{-/-}$ mice	Genetic	Resembles human disease		PBC	[29]
NOD.c3c4 mice	Genetic	Resembles human disease	Injury of the extrahepatic biliary ducts	PBC	[31]
3,5-Diethoxy-carbonyl-1,4-dihydrocollidine (DDC)	Feeding	Resembles human disease		Sclerosing cholangitis with oval cell activation	[33]
α-Naphthylisothiocyanate (ANIT)	Feeding	Fast		Cholestatic fibrosis	[34]
CCl_4 treatment	Injection, oral	Highly reproducible, fast, resembles properties of human fibrosis, good comparability due to abundant reference studies	Enhanced mortality by oral application	Toxic fibrosis	[43]
Thioacetamide (TAA) treatment	Injection, feeding	Injection, fast	Feeding, long latency	Toxic fibrosis and hepatocellular carcinoma (HCC)	[49-51]
Dimethylnitrosamine (DMN)	Injection	Fast	Mutagenic and carcinogenic	Toxic fibrosis and HCC	[58]
High-fat diet	Feeding	Fast, resembles features of insulin resistance and metabolic syndrome		Steatohepatitis and subsequent fibrosis	[70,71]
Lieber-DeCarli diet	Feeding	Well-tolerated	Long latency, only mild injury	Alcohol-induced liver fibrosis	[73]
Methionine- and choline-deficient (MCD) diet	Feeding	Fast, strong steatohepatitis along with elevated TNF	Metabolic profile only partially reflects human NASH, no insulin resistance, body weight loss, different outcome in different mouse strains	NASH-associated fibrosis	[76,77,83,84]
CD (solely choline-deficient) diet	Feeding	Resembles sequence steatosis -inflammation - fibrosis		NASH-associated fibrosis	[88]
Choline-deficient, ethionine-supplemented (CDE) diet	Feeding	Stronger NASH development compared to CD, activates hepatic progenitor cells		NASH-associated fibrosis	[90]
ob/ob mice	Genetic		Does not progress spontaneously to NASH or fibrosis	Fatty liver disease	[91]
Diethylnitrosamine (DEN) treatment	Injection	High HCC incidence, highly reproducible, well-tolerated, not associated with serious side effects	No development of fibrosis	Resembles human HCC associated with poor prognosis	[184]

Figure 3. Overview of mouse models of liver fibrosis. (From *Liedtke C et al.* Fibrogenesis Tissue Repair. 2013)

One of the most established animal models of cholestatic liver disease and fibrosis comprises genetic deletion of the Mdr2 gene in mice [13]. There are several independent reports that have shown that mutations or polymorphisms in the human MDR2 homologue are associated with different entities of cholestatic liver disease in patients [13-15], which in turn underlines the benefit of animal models for clinical fibrosis research. As regards to nutritional models, different methods in which the pathophysiology of liver fibrosis is induced via special diets are described (for example α-Naphthylisothiocyanate (ANIT) [16], high-fat diet, Lieber-DeCarli diet, Methionine and cholinedeficient (MCD) diet, CD (solely cholinedeficient) diet, Choline-deficient, ethioninesupplemented (CDE) diet. The high-fat diet resembles features of insulin resistance and metabolic syndrome and it is well-tolerated [17,18] ; Methionine and cholinedeficient (MCD) diet is responsible of NASH-associated fibrosis with elevated values of Tumor Necrosis Factor (TNF-α) [20-22]. CD (solely cholinedeficient) diet resembles sequence steatosis-inflammation-fibrosis with a

NASH-associated fibrosis [23]. Lieber-DeCarli diet determines alcohol-induced liver fibrosis with long latency [19].

Others experimental hepatic models of hepatic fibrosis are represented by porcine serum [24] and Concanavalin A (ConA) injection [25]. This latter involves immunological responses, such as lymphocyte infiltration and IFN-γ synthesis, compatible for human cases.

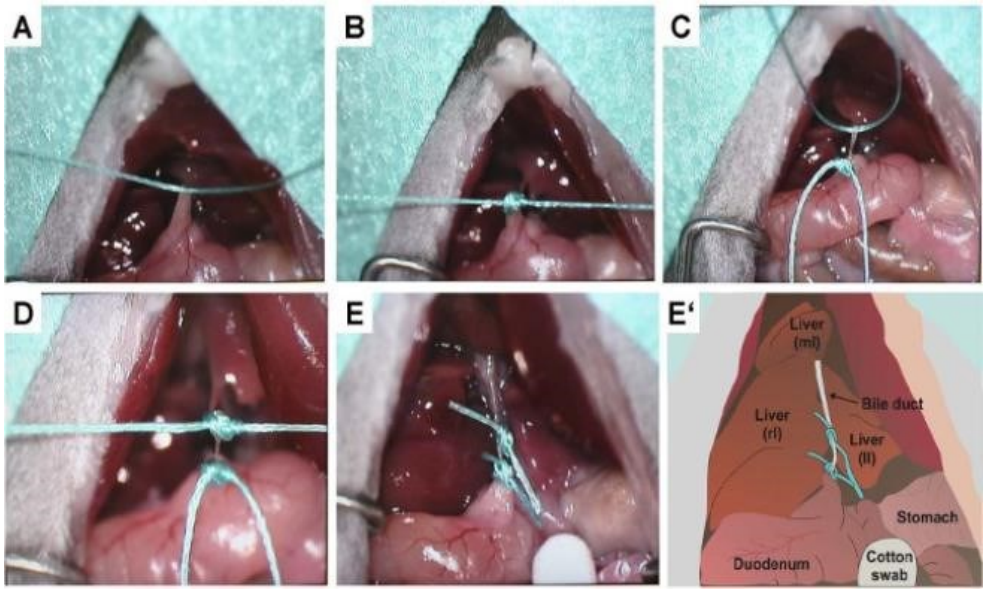


Figure 4. Representation of surgical model of bile duct ligation. (From *Tag CG et al.* J Vis Exp. 2015)

Despite the different models of liver fibrosis described over the years, an appropriate animal model with essential similarities to patients with chronic hepatitis should be established, in order to better elucidate the underlying mechanisms. Ideally, the animal model should have the following features : i) it should be based upon immunological mechanisms ; ii) hepatic fibrosis should be induced following continous liver injury ; iii) the inducer of liver fibrosis should not be a direct hepatotoxic agent. However, no animal model that satisfies these three criteria has been described and established until now. ConA-induced hepatitis is characterized by immunological mechanisms and it presents many similaritis to chronic hepatic disease in humans. For this reason, ConAinduced hepatitis models was thought to be one of the best models to induce liver fibrosis mediated by hepatocellular injury [26,27].

Concanavalin A (ConA) is a lectin (carbohydrate-binding protein) originally extracted from the jack-bean, *Canavalia ensiformis*, a member of the legume lectin family (Figure 5).

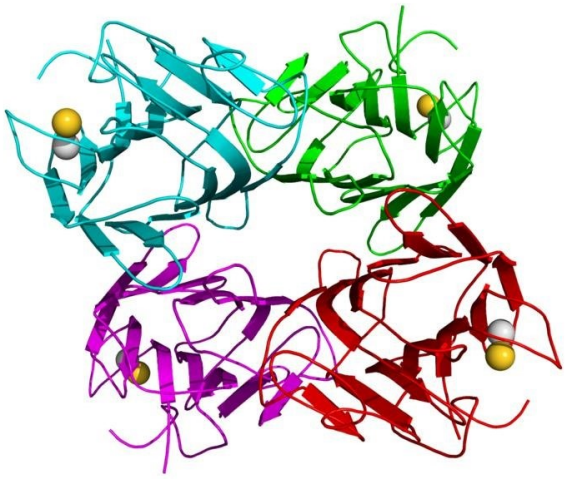


Figure 5. Crystallographic structure of Concanavalin A based on the Protein Data Bank.

It is known that the plasma membrane of hepatocytes strongly binds ConA. Furthermore, ConA has shown to have great binding affinity to insulin receptors in liver tissue. It has also been shown as a a strong lymphocyte mitogen and a stimulator of several matrix metalloproteinases (MMPs). The ultrastructural analysis found that an early event in liver damage is represented by the adhesion of leukocytes to endothelial cells, followed by disruption of the endothelial epithelium and finally by hepatocyte necrosis.

Pathogenesis of liver fibrosis

A key role in fibrogenesis process is played by hepatic stellate cells (HSC), also known as perisinusoidal cells or Ito cells (earlier *lipocytes* or *fat-storing cells*) located in the perisinusoidal space of the liver, the so-called Disse's spaces (a small area between the sinusoids and hepatocytes). In normal liver, stellate cells are described as being in a *quiescent* state. Quiescent stellate cells represent 5-8% of all human liver cells [28].

When the liver is damaged, stellate cells can change into an *activated* state and they go through a process of transdifferentiation, by trasforming in myofibroblasts with capacity of proliferation, contractility, and chemotaxis, as shown in *Figure 6*. Moreover, the activated hepatic cells are

responsible for the accumulation of ECM proteins which distorts the hepatic architecture by forming a fibrous scar, and the subsequent development of nodules of regenerating hepatocytes defines liver cirrhosis. Cirrhosis produces hepatocellular dysfunction and increased intrahepatic resistance to blood flow, which result in hepatic insufficiency and portal hypertension, respectively.

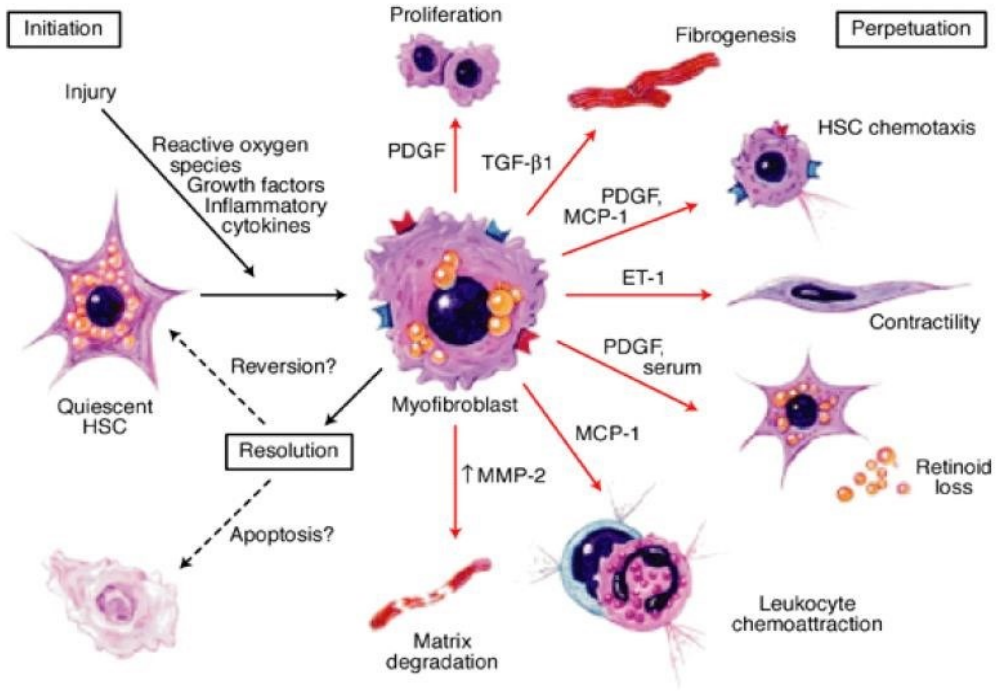


Figure 6. Schematic representation of activation of HSCs and the role of different cytokines in fibrogenesis process.

However, the most powerful proliferative stimulus of stellate cells is represented by PlateletDerived Growth Factor (PDGF), which acts by binding to a trans-membrane receptor (PDGFR) with tyrosine-kinase activity [29]. The PDGF signaling pathway is very complex, as shown in *Figure 7*. Two types of PDGFRs have been identified : alpha-type and beta-type PDGFRs. PDGFRbeta is a key marker of hepatic stellate cell activation in the process of fibrogenesis. The PDGF is responsible for the massive proliferation of HSCs after liver injury and stellate cells chemotaxis. The PDGF family also includes a few other members of the family, including the Vascular Endothelial Growth Factor (VEGF) sub-family. In physiological conditions, PDGF is primarily expressed in the α granules of platelets. However, when liver damage occurs, PDGF may be highly expressed in macrophages, injured endothelial cells and activated HSCs. During the early stages of various chronic liver diseases, increased PDGFR expression on the membranes of activated HSCs enhances cellular chemotaxis and

decreases the amount of intracellular vitamin A, demonstrating that PDGF is involved in ECM production [30].

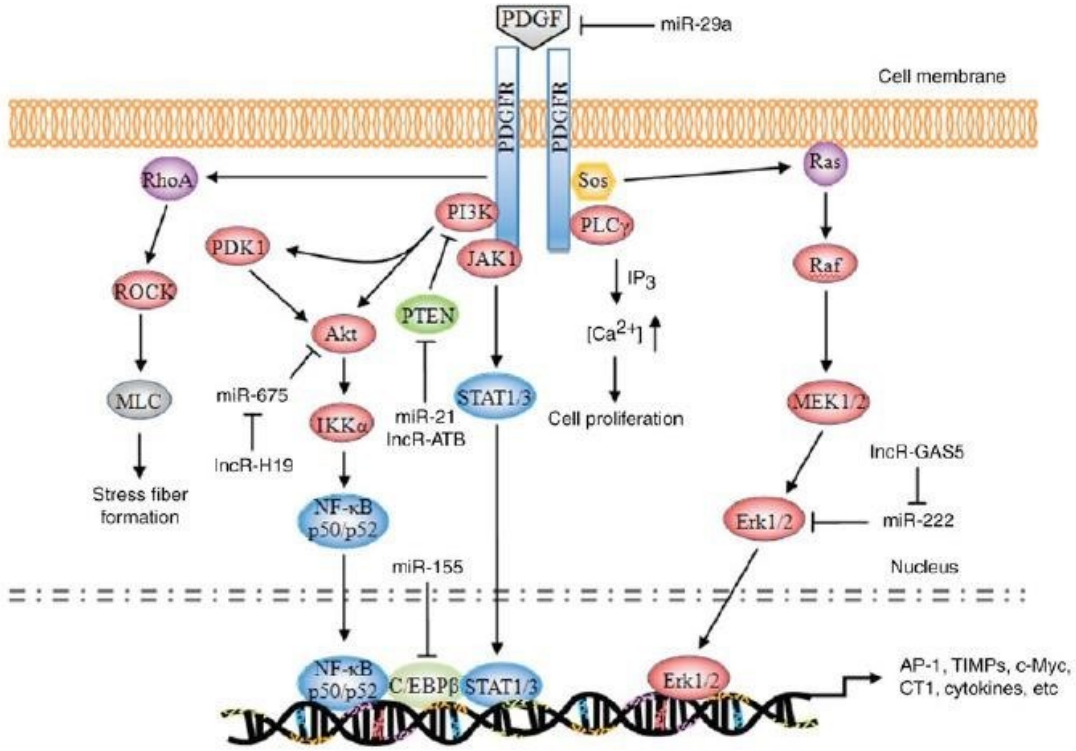


Figure 7. Schematic representation of the PDGF signaling pathway.

PDGF, platelet‐derived growth factor; PDGFR, PDGF receptor; miR, microRNA; PI3K, phosphatidylinositol 3‐kinase; PLCγ, phospholipase Cγ; Sos, son of sevenless homolog; JAK1, tyrosine‐protein kinase JAK1; PDK1, 3‐phosphoinositide‐dependent protein kinase 1; Akt, RAC‐α serine/threonine protein kinase; PTEN, phosphatidylinositol 3,4,5‐trisphosphate 3‐phosphatase and dual‐specificity protein phosphatase PTEN; ROCK, Rho‐associated protein kinase 1; MLC, membrane protein MLC; NF‐κB, nuclear factor‐κB; IKKα, inhibitor of NF‐κB kinase subunit α; C/EBPβ, CCAAT/enhancer‐binding protein β; STAT, signal transducer and activator of transcription; MEK, dual specificity mitogen‐activated protein kinase kinase; Erk, extracellular signal‐regulated kinase; AP‐1, transcription factor AP‐1; TIMP, metalloproteinase inhibitor; c‐Myc, myc proto‐oncogene protein; CT1, cardiotrophin 1.

Recently, several drugs inhibiting PDGFR have been synthesized, among these *Imatinib* (also termed Gleevec® and STI-571), a selective tyrosine kinase inhibitor (TKI), which is able to specifically target PDGFR, Abl, Abelson tyrosine‐protein kinase 2, mast/stem cell growth factor receptor, and their oncogenic forms Bcr‐Abl [31]. Imatinib has been demonstrated to induce HSC apoptosis in vitro and

to control progression and perpetuation of liver fibrosis in CCl4- and thioacetamide (TAA)-treated mice, ConA-induced hepatitis and bile duct ligation [32-34]. Nowadays, Imatinib is largely used in clinical practice for the treatment of chronic myeloid leukemia and gastrointestinal stromal tumors c-kit positive [35,36]. *Sorafenib* (also termed Nexavar®) is a first‐line oral chemotherapy drug for patients with advanced hepatocellular carcinoma. As an RTK inhibitor, sorafenib may target a number of kinases on the cytomembrane, including Raf, VEGFR2/3, and PDGFR‐β. Sorafenib has previously been demonstrated to be a potential antifibrotic agent, due to its multi‐targeting of the Ras/MEK/ERK pathway [37].

Recent studies have also shown that *carbon monoxide (CO)*, despite its limitations as a toxic gas, has potential therapeutic effects in animal models of pulmonary fibrosis [38,39]. CO is physiologically produced by the activity of heme-oxygenases during heme catabolism, and it seems to mediate the anti-inflammatory effects of heme-oxygenase (HO-1) [40]. Heme oxygenase 1 is one of the main effectors of nuclear factor erythroid 2-related factor 2 (Nrf2)-dependent cell responses. HO-1 is the inducible form of heme oxygenase, the first rate-limiting enzyme in the degradation of heme into biliverdin/bilirubin, carbon monoxide (CO), and free iron. HO-1 expression is induced by a wide variety of stimuli, including its substrate, as well as pro-inflammatory cytokines, heavy metals, nitric oxide, UV and ROS, therefore playing a key role in the maintenance of cellular homeostasis.

The *CORMs* (Carbon Monoxide Releasing Molecules) represent a new class of molecules able to release CO in cellular systems in order to mimic the local action of the HO-1 [41]. Carbon monoxide (CO) is emerging as an important and versatile mediator of physiological processes and it has beneficial effects in a range of vascular and inflammatory-related disease models.

The recent discovery that certain transition metal carbonyls function as CO-releasing molecules (CO-RMs) in biological systems highlighted the potential of exploiting this and similar classes of compounds as a stratagem to deliver CO for therapeutic purposes.

CORMA1 is one of the most important pharmacologically active carbon monoxide-releasing molecule. It presents different bioactive properties such as : mediating cerebral vasodilatation, smooth muscle relaxation of internal anal sphincter, suppression of smooth muscle cell proliferation, angiogenesis, inhibition of apoptosis and cardiac hyperthrophy, improved renal function, and anti-inflammatory and immunological responses [42,43].

In a previous study [43], we have evaluated the role of the anti-inflammatory pathway Nrf2/HO1/CO and determined whether the in vivo administration of CORM-A1 influences serological and

histological development of Con-A-induced hepatitis in a murine model of autoimmune hepatitis. This study was conducted in order to understand further the immunoregulatory mechanisms operating in the development and regulation of ConA-induced hepatitis in mice. The administration of CORM-A1 determined an improvement in several sero-immunological and histological parameters, as shown in *Figure 8*.

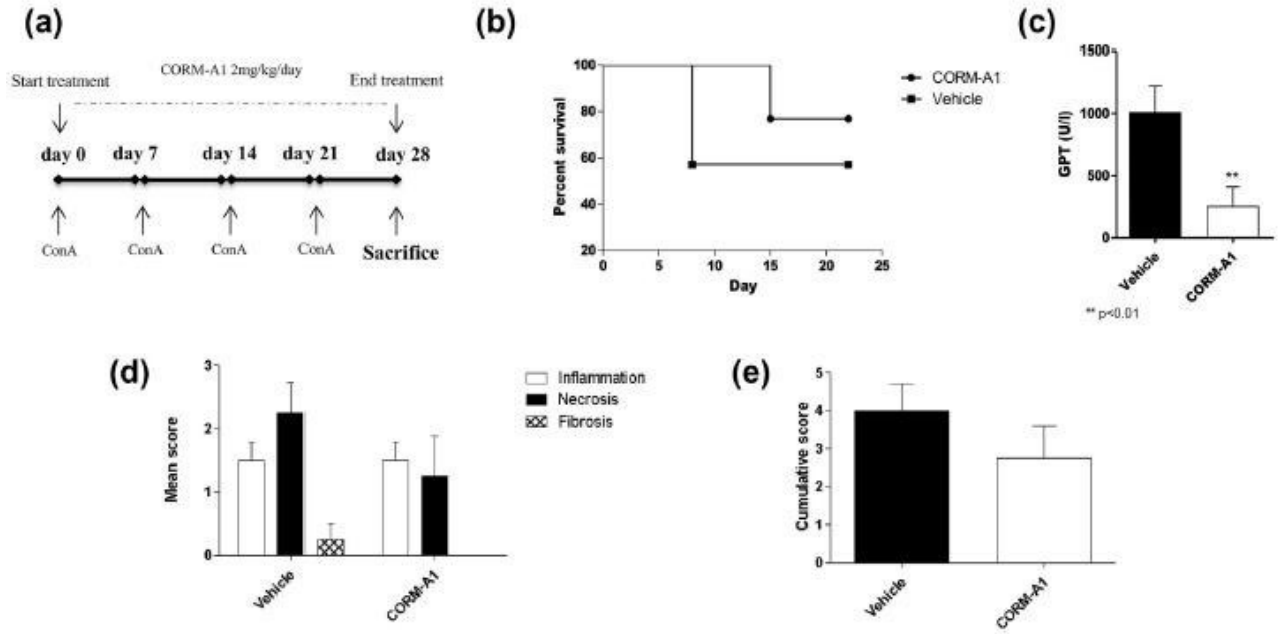


Figure 8. Experimental layout of the chronic model of Concanavalin A-induced hepatitis (a); Kaplan-Meier curve showing survival in vehicle-treated and CORM-A1-treated mice during the course of the experimental period (b); circulating transaminase levels in the serum of vehicletreated and CORM-A1-treated mice upon the second Concanavalin A challenge (day 8) (c). Results from the histopathological evaluation of liver of mice sacrificed one week after the fourth Concanavalin A administration (d); cumulative score of the histopathological evaluation (e);

The aim of the present study was to test the efficacy of new drug therapies 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 analyzed and studied the effectiveness of the PDGF-inhibitor Imatinib, of the angiogenesis inhibitor - Sorafenib, administered alone or in combination, and of the carbon monoxide release - CORM-A1, 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.

EXPERIMENTAL STUDY

Animals

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 used in the experiment complies with the 86/609/EEC directive, reaffirmed by the Italian Legislation D.L. No 116 of 27 January 1992. The material for the maintenance and care of the animal is in accordance with the rules of the Executive Council of the EEC 86/609. The experimental study was approved by the Institutional Animal Care and Use Committee (IACUC) of University of Catania.

Induction of liver fibrosis and experimental treatments

The experimental design is shown in *Figure 9*. The protocol treatment is summarized in *Table 1*.
The control group consists of animals that received the 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 4 consecutive weeks. 24h after each ConA injection transaminase levels (GPT) in serum samples were evaluated by Reflotron® analyzer. One week after the fourth administration of ConA, the treatment protocol started. All treatments were performed 5 times a week for 3 consecutive weeks.

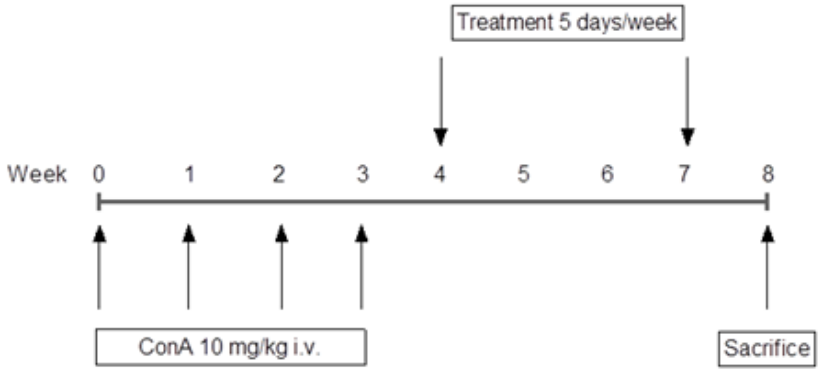


Figure 9. Experimental study design

Group	N° animals	Treatment	Dose	Administration
A	15	Sham	-------	----------
B	15	Vehicle	100 □l	p.o.
C	15	Glivec	50 mg/Kg	p.o.
D	15	Nexavar	30 mg/kg	p.o.
E	15	Nexavar + Glivec	30 mg/Kg + 50 mg/kg	p.o.
F	15	CORM-A1	2 mg/kg	i.p.

Table 1. Treatment schedule.

p.o.: per os, orally i.p.: intra-peritoneal

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) or Masson's Trichrome to evaluate the degree of liver fibrosis and inflammation/necrosis.

The *METAVIR score* [44] was used to quantify the liver fibrosis severity index, as follows:

- F0, absence of fibrosis;
- F1, portal fibrosis without fibrous septa;
- F2, portal fibrosis with rare fibrous septa;
- F3, numerous fibrous septa, not yet cirrhosis;
- F4, cirrhosis

In *Figure 10* a schematic comparison between Ishak and METAVIR scores in liver fibrosis staging is shown.

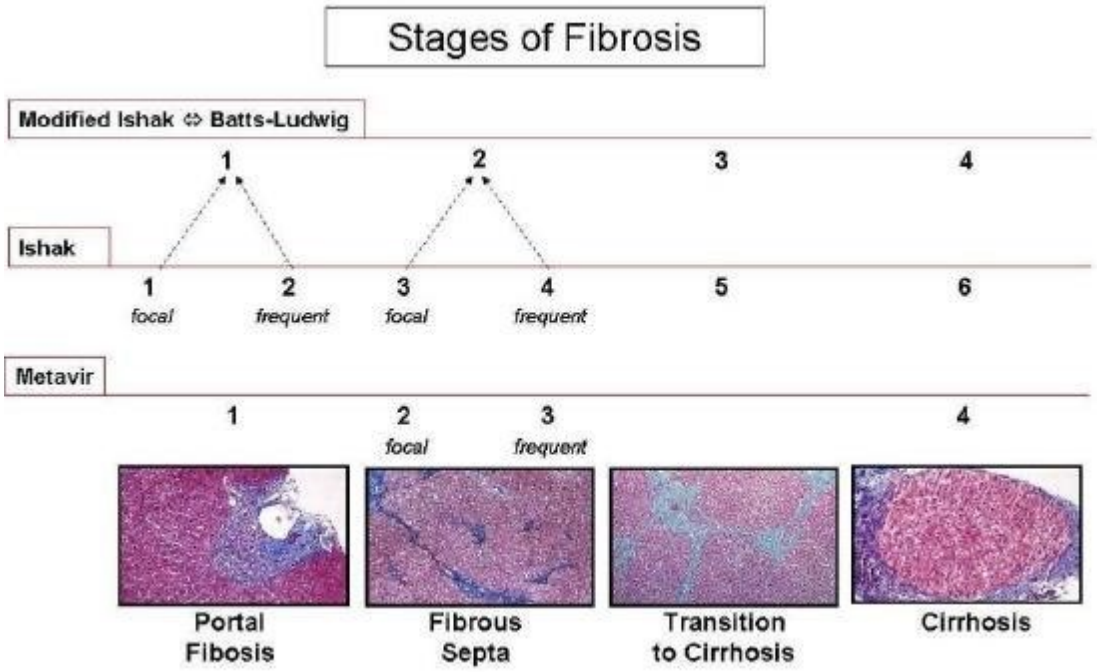


Figure 10. Comparison of Ishak and METAVIR score in liver fibrosis staging.

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.

Quantitative RT-PCR (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 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 retrotranscribed 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).

The sequences of the primers used were the following :

- ✓ ACTA2 forward: GTCCCAGACATCAGGGAGTAA; ACTA2 reverse: TCGGATACTTCAGCGTCAGGA;
- ✓ interleukin-6 (IL-6) forward: TAGTCCTTCCTACCCCAATTTCC; IL6 reverse: TTGGTCCTTAGCCACTCCTTC;
- ✓ TGF-beta2 forward: TCGACATGGATCAGTTTATGCG; TGF-beta2 reverse: CCCTGGTACTGTTGTAGATGGA;
- ✓ COL2A1 forward: GGTGAGCCTGGTCAAACGG; COL2A1 reverse: ACTGTGTCCTTTCACGCCTTT;
- ✓ TIMP1 forward: GCAACTCGGACCTGGTCATAA; TIMP1 reverse: CGGCCCGTGATGAGAAACT
- ✓ Beta-actin forward: CATCATGAAGTGTGACGTTGAC; Beta-actin reverse: GCATCCTGTCAGCAATGCC.

The level of gene expression, normalized towards the control, was calculated using the following formula:

$$2^{-\Delta\Delta Ct}$$

where $2^{-\Delta\Delta Ct}$ = (Ct, target gene -Ct, beta-actin) treated group - (Ct, target gene -Ct, beta-actin) control group.

The statistical analysis was performed using the one-way-ANOVA test. The kinetics of mortality was calculed by Mantel-Cox test. A p-value <0.05 was considered statistically significant.

Results

Effects of ConA injection

Administration of ConA (10 mg/kg) was associated with about 35% mortality, with a peak incidence between the second and third week, as reported in *Table 2*. The remaining animals were randomized to obtain a homogeneous number of animals for each experimental group. All animals included in the study showed significant increases in transaminases levels during the period of ConA administration, as shown in *Figure 11*.

Kinetics of Mortality					
	T1	T2	T3	T4	Log-rank (Mantel-Cox) Test
Glivec	0	1	2	0	0,1963
Nexavar	0	4	1	0	0,688
Glivec + Nexavar	0	1	3	0	0,337
CORM-A1	0	0	3	0	0,1988
Vehicle	0	6	0	0	

Table 2. Kinetics of mortality after ConA injection in the experimental model.

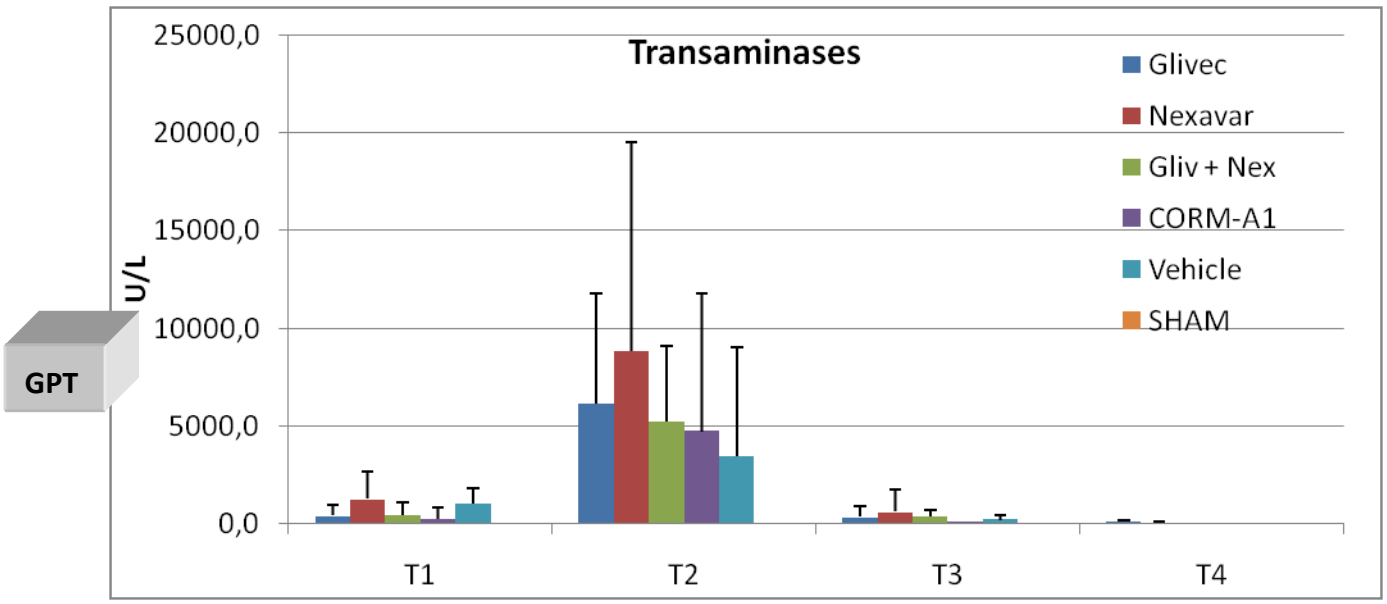


Figure 11. GPT levels 24h after each ConA injection. Showing the peak at second week.

- *Effects of Glivec, Nexavar and CORM-A1 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. 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 cease to proliferate and start to synthesize large amounts of extracellular component proteins. Expression analysis of alpha-SMA showed a 37% increase in the group of vehicle-only animals, compared to Sham animals. Treatments with Glivec and Sorafenib were associated with a reduction in alpha-SMA levels of 51% and 34%, respectively, compared to the vehicle ($p=0.89$ and $p=0.98$, respectively). No additive or synergistic effects were observed between Glivec and Sorafenib because, in the group of animals treated with both drugs, alpha-SMA levels were found to be reduced by 40% compared to the vehicle ($p=0.96$).

Treatment with CORM-A1 was associated with a 49% reduction in alpha-SMA compared to the vehicle ($p=0.96$). All results relative to the expression analysis of alpha-SMA are summarized in *Figure 12*. Data are shown as normalized mean ± SD.

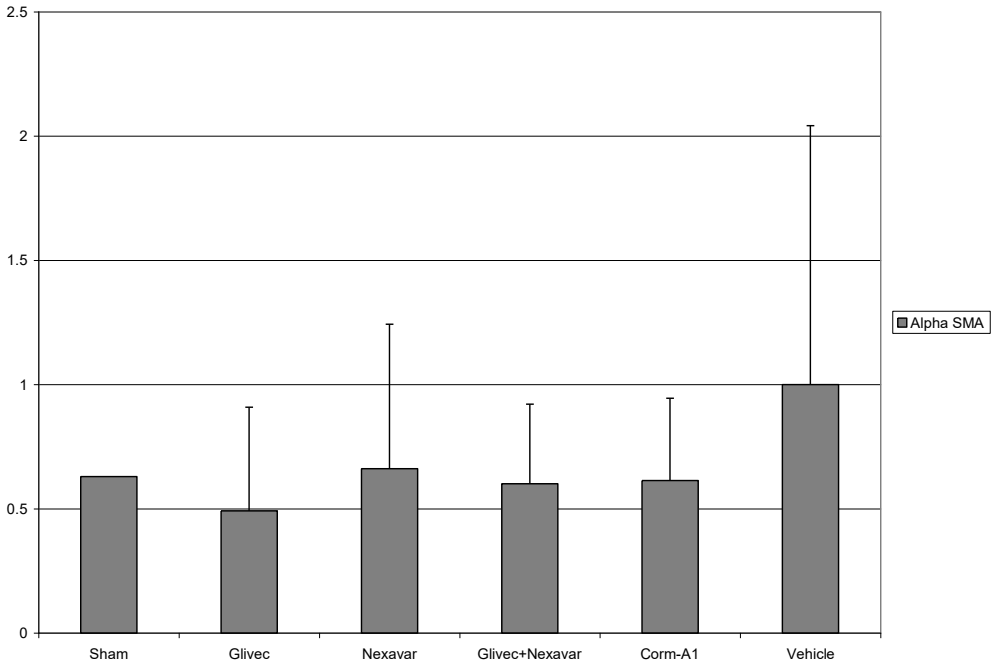


*Figure 12***.** Effects of Glivec, Nexavar and CORM-A1 on the expression of alpha-SMA.

- *Effects of Glivec, Nexavar and CORM-A1 on the expression of COL1A2*

Collagen alpha-2 (I) chain is a protein that in humans is encoded by the *COL1A2* gene. This gene encodes one of the chains for type I collagen, the fibrillar collagen found in most connective tissues. Mutations in this gene are associated with osteogenesis imperfecta, Ehlers Danlos syndrome, idiopathic osteoporosis, liver fibrosis and atypical Marfan syndrome.

During the fibrogenesis process, the hepatic myofibroblasts represent the major source of fibrillar collagen Type I in fibrotic liver. The analysis of COL1A2 expression showed an increase of 46% in the vehicle group compared to Sham animals. Treatments with Glivec and Sorafenib resulted in a reduction in COL1A2 levels of 38% and 44%, respectively, compared to the vehicle ($p=0.72$ and $p=0.60$, respectively). The association Glivec-Sorafenib was associated with a 37% reduction in COL1A2 levels compared to vehicle group ($p=0.74$).

Conversely, the treatment with CORM-A1 did not have any modulating effect on the levels of COL1A2 expression (reduction of only 3% compared to the vehicle group), as shown in *Figure 13*.

- *Effects of Glivec, Nexavar and CORM-A1 on the expression of Transforming Growth Factor-beta2* (*TGFb2)*

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 in humans. In chronic liver diseases, TGF-β signaling participates in different stages of disease progression, from acute liver injury toward fibrosis, cirrhosis and cancer. When a chronic injury takes place, mobilization of lymphocytes and other inflammatory cells occur, 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, the analysis of TGFbeta2 expression levels showed an increase of 13% in the vehicle group compared to Sham animals. Treatments with Glivec and Sorafenib resulted in a reduction in TGFbeta2 levels by 21% and 35%, respectively, compared to the vehicle (p=0.96 and p=0.81, respectively). The association between Glivec and Sorafenib did not modulate TGFbeta2 levels compared to the vehicle group.

However, treatment with CORM-A1 was associated with an increase of 60% in TGFbeta2 levels compared to the vehicle group (p=0.24). These results are reported in the *Figure 13*.

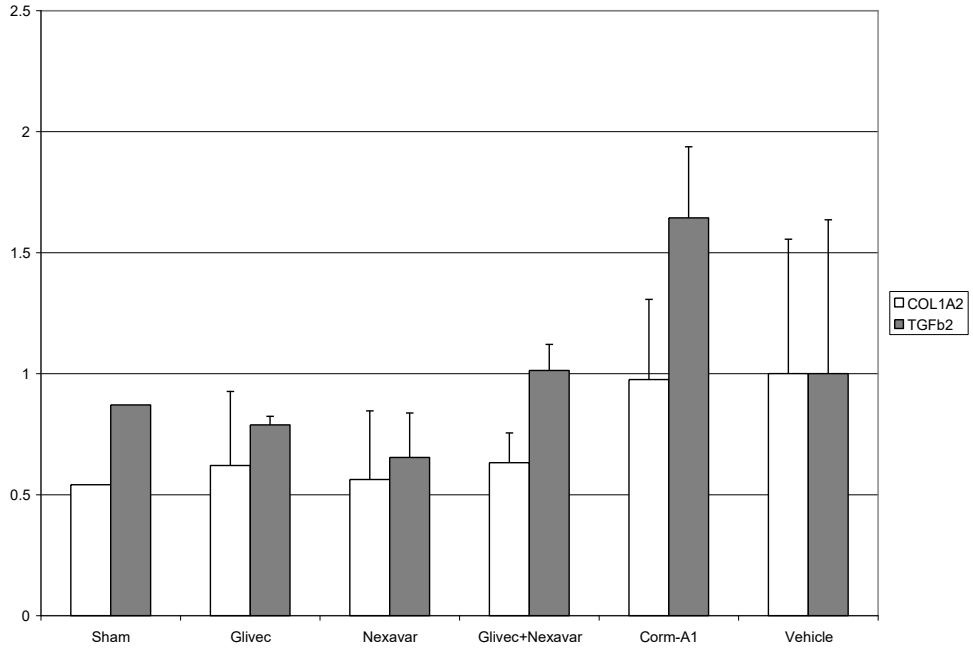


Figure 13. Effects of Glivec, Nexavar and CORM-A1 on the expression of COL1A2 and TGFb2.

- *Effects of Glivec, Nexavar and CORM-A1 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 pro-fibrotic properties and it is also involved in liver regeneration after hepatectomy [45]. The vehicle group showed an IL-6 increase of 52% compared to Sham group. Treatment with Glivec reduced IL-6 levels by 75% (p=0.36), while Sorafenib and the Glivec-Sorafenib combination resulted in a mild increase of this cytokine level in the liver tissue (15% and 47% respectively) compared to vehicles. Moreover, treatment with CORM-A1 increased IL-6 levels by 65% compared to the vehicle group (p=0.5), as shown in *Figure 13*.

- *Effects of Glivec, Nexavar and CORM-A1 on the expression of Tissue Inhibitor MetalloProteinases 1 (TIMPs)*

As suggested by their name, 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. Although hepatic stellate cells (HSCs) clearly play a role in matrix protein synthesis, they also are 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 sham. Treatments with Glivec and Sorafenib reduced TIMP1 levels by 26% and 30%, respectively, while in the group treated with the combination of the two afore mentioned drugs a 20% reduction was observed.

Treatment with CORM-A1 increased TIMP1 levels by 4.6 times compared to vehicle group, as shown in *Figure 14*.

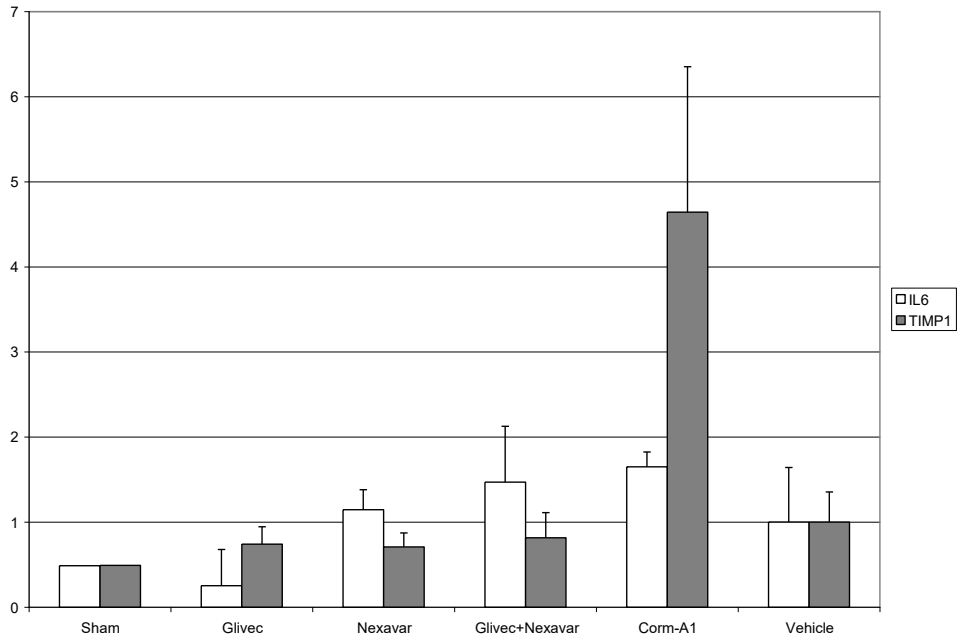


Figure 14. Effects of Glivec, Nexavar and CORM-A1 on the expression of IL-6 and Tissue Inhibitor MetalloProteinases 1 (TIMPs).

Histopathological analysis

At histological analysis treated groups with Glivec, Sorafenib and the association Glivec-Nexavar had significantly less histological evidence of liver fibrosis compared to vehicle group using METAVIR scoring of H&E stained liver sections. Inflammation and necrosis index were also reduced in treated groups compared to vehicle group. All histological results are summarized in *Table 2*.

	INFLAMMATION	NECROSIS	FIBROSIS
VEHICLE (n. 9)	1.6	2.6	1.8
GLIVEC (n. 12)	1	1	0
SORAFENIB (n. 12)	1	1	0
CORM A1 (n. 12)	2.1	1	0
GLIVEC + NEXAVAR (n. 11)	1	1.6	0

Table 2. Median values of inflammation, necrosis and liver fibrosis score at histological analysis.

The morphology of the liver in the control and ConA-treated groups are represented in *Figure 15*.

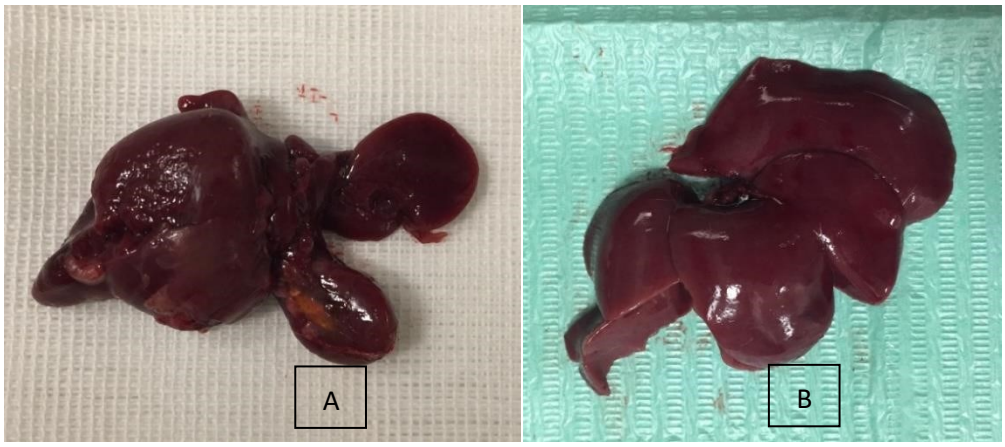


Figure 15. Liver samples after animals' sacrifice. A) ConA group : the alteration of the hepatic architecture and macroscopic morphology is largely visible ; B) Sham healthy group.

In *Figures 16-17* some histological pictures of liver fibrosis and peri-portal inflammation with areas of hepatic necrosis by ConA are shown.

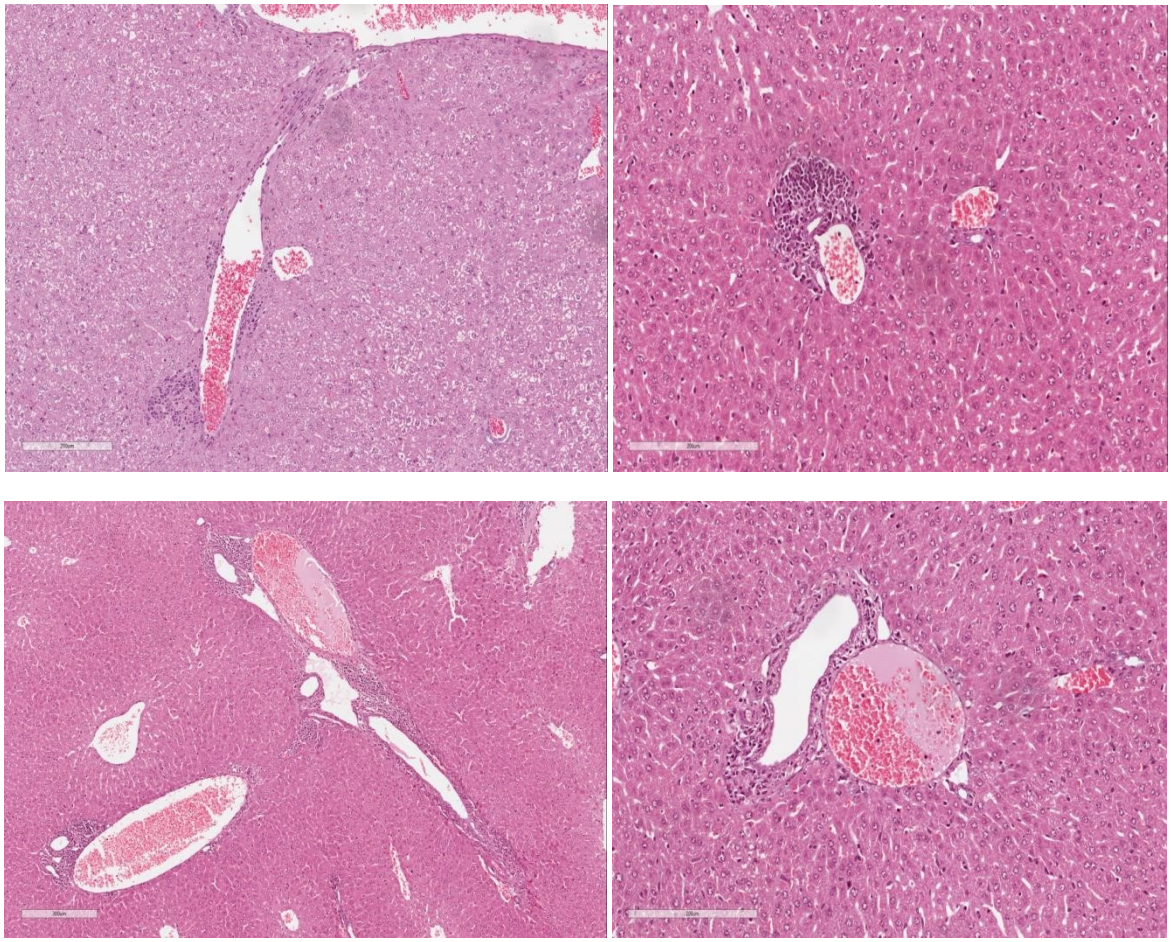


Figure 16. Examination of liver histology using H&E staining (x400) showed liver fibrosis F1 and peri-portal inflammation.

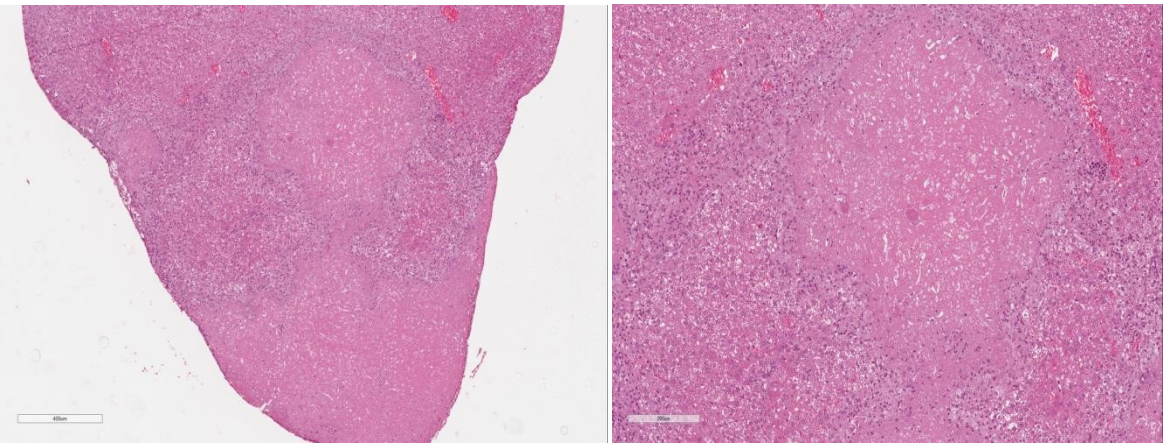


Figure 17. H&E staining (x400) showing hepatic necrosis by ConA.

Discussion

In the current study we have evaluated the anti-fibrotic effect of Imatinib (Glivec) and Sorafenib (Nexavar), administered alone or in combination, and of CORM-A1 in a pre-clinical model of autoimmune hepatitis in mice by ConA injection. Several independent studies have already shown that Imatinib, through its inhibitory effect on PDGFR, plays an important anti-fibrotic role both *in vitro* and *in vivo* analysis. Similarly, Sorafenib, a double inhibitor of VEGFR/PDGFR and RAF/MEK/ERK pathways, has been studied for its anti-fibrotic effects in pre-clinical models of chronic liver disease.

In our study, the liver expression levels of *alpha-SMA* (ACTA2) and fibrillar collagen *COL1A2* were determined *ex-vivo*, as well as the pro-fibrogenetic cytokines *TGFbeta2* and *IL-6*. Finally, the expression levels of the metalloproteinase inhibitor - *TIMP1* were evaluated.

The obtained results suggest that treatments with Imatinib and Sorafenib play a similar and overlapping effect in inhibiting the main molecules involved in the process of liver fibrosis, even if not statistically significant. An important aspect from an experimental point of view 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 Concanavalina A. This differentiates this analysis from other precedent studies.

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. However, in the current model, the mortality peak and transaminase levels was observed between the second and third week after ConA injection, and transaminases levels reduced within the normal range before starting the pharmacological treatment.

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 Glivec + Sorafenib compared to the groups treated with the two drugs alone. Moreover, we could speculate that the antifibrotic effect is not modulated by the inhibition of the Raf/MEK/ERK pathway. However, *gainof-function* and *gene knockdown* studies on specific liver subpopulations, in particular, hepatic stellate cells 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.

Contrary to what has been observed for Imatinib and Sorafenib, treatment with CORM-A1, although it resulted on reduction in alpha-SMA levels, it was nevertheless associated with an increase in the profibrogenic factors TGFbeta2, IL6 and TIMP1. This results are in agreement with the data of *Wang et al.* [46], which showed that HO-1 inhibition causes a reduction in fibrosis and TGFbeta and TIMP-1/MMP levels -2 in a rat BDL model.

Based on these data, CORM-A1 does not seem to find possible application as an anti-fibrotic drug. However, given the important inflammatory properties of CORM-A1, reported in several independent studies [43, 47,48], its potential hepatoprotective effect should be investigated in all chronic liver diseases having an immuno-inflammatory etiopathogenesis. In this context, the CORM-A1 may be applied in prophylaxis regimen in order to delay the evolution towards fibrosis and hepatocellular carcinoma in patients with chronic liver disease.

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/macroscopic structure.

Ongoing clinical trials

The growing progress in understanding of the molecular mechanisms and pathways of hepatic fibrosis has enabled researchers to identify several promising molecular targets for antifibrotic treatments. A summary of the clinical trials with liver fibrosis as primary endpoint is provided in *Tables 3a/3b*.

Recent clinical trials with efficient causal therapy have demonstrated reversibility of advanced liver fibrosis. Perhaps the best example is a study of 348 patients with chronic hepatitis B who were treated with the potent antiviral tenofovir. After five years, regression of fibrosis was observed in 91% of patients with significant fibrosis at study entry. Only 12 of 252 patients (5%) showed fibrosis progression, while 71 of the 96 patients (74%) with cirrhosis at baseline were no longer cirrhotic at year five [49]. In scientific literature there are many clinical studies with liver fibrosis as primary endpoint : for example the administration of PPARγ agonists (e.g. glitazones) and pentoxifylline (anti-TNF) decreased fibrosis progression and improved hepatic steatosis, lobular inflammation and fibrosis respectively in patients with NASH [50,51]. There are also drugs in clinical trials for other indications that have antifibrotic potential for liver fibrosis such as antiTGFβ, anti-IL4 and anti-IL-13 [52]. The renin–angiotensin system (RAS) is implicated in liver fibrogenesis and portal hypertension. Activated HSCs produce angiotensin II, which is crucial for the development of liver fibrosis. Accordingly, the inhibition of RAS, via angiotensin II antagonists, such as losartan and olmesartan, attenuates liver fibrosis development in animals [53].

Although the TGFb pathway has a key role in the fibrotic process, the development of drugs targeting this pathway is still in its infancy. Only pirfenidone, an orally available pyridine derivative that inhibits TGFb production, has been tested in a small study in HCV-related fibrosis, showing to improve in the long-term both liver inflammation and fibrosis [54].

The anticoagulants warfarin and ximelagatran are currently in a Phase II study aiming to demonstrate improvements in surrogate markers of liver fibrosis over a short period of anticoagulation (NCT00180674).

Endogenous opioids have been shown to present profibrogenic activity and opioid antagonism has been tested in animal models [55,56].

Finally, the possibility of using stem cells to treat liver diseases has been extensively explored in preclinical and clinical studies, with encouraging results [57,58]. At present, five Phase II trials using mesenchymal stem cells in patients with liver cirrhosis are listed on ClinicalTrials.gov under the identifiers : NCT01741090, NCT00476060, NCT01728728, NCT01877759, and NCT01854125.

Monocytes, which play a key role in inflammation and fibrosis, are also precursors of fibrocytes, macrophages, and dendritic cells and share characteristics with myeloid suppressor cells. At the interface of innate and adaptive immunity, monocytes help orchestrate adaptive immune responses, with proinflammatory monocytes promoting fibrogenesis. Chemokines and their receptors are important in monocyte recruitment and activation, representing attractive targets for fibrosis modulation. There are two different classes of macrophages. M1 macrophages are induced by IFN or IL-12, while IL-4, IL-13, and GM-CSF induce M2 macrophages. Macrophages appear to be fibrogenic during fibrosis progression and fibrolytic during its reversal, but a detailed functional analysis and assignment to M1 or the various M2 subclasses has remained elusive. While M1 macrophages are activated in immediate defense against pathogens or detrimental cellular debris, M2 macrophages are generally thought to promote wound healing (i.e., fibrogenesis) and immune suppression (e.g. facilitating cancer growth as tumor-associated macrophages). M2 macrophages respond to IL-4 and IL-13 via IL-4 receptor and IL-13 receptor α1 (with IL-13 receptor α2 serving as negative regulator) [49, 59,60]. The targeting of macrophage polarization in liver inflammation and fibrosis represents an attractive therapeutic option. However, the polarization of M1/M2 macrophages in autoimmune hepatitis and liver fibrosis remains unclear.

Myeloid-derived suppressor cells (MDSCs) represent another heterogeneous population of cells that inhibit the proliferation and regular functions of T cells, suppress the cytotoxicity of NK cells, and accelerate the polarization of regulatory T cells. In a recent paper [26], the authors have studied the effects of splenectomy in a mouse model of Concanavalin A-induced liver fibrosis. They found that splenectomy promotes macrophage polarization to M2 and it may suppress the progression of liver fibrosis.

A deep understanding of the cellular and molecular mechanisms underlying liver fibrosis has enabled the development of 'pathogenetic tailored' therapeutic interventions [61,62].

Drug	Sponsor/Collaborator	Disease	Phase	NCT identifier/Refs
PPARγ agonists				
Farglitazar	GlaxoSmithKline	HCV	II	NCT00244751/[56]
Rosiglitazone	Association pour la Recherche sur les Maladies Hépatiques Virales	NASH	II	NCT00492700
Pioglitazone	University of Florida/The University of Texas at San Antonio	NASH; NAFLD; T2DM	IV	NCT00994682
	National Institute of Allergy and Infectious Diseases	HCV; liver disease; fatty liver; steatosis	IV	NCT00742326
	University of Michigan	HCV	IV	NCT00189163
Saroglitazar; pioglitazone	Command Hospital, India	Fatty Liver	III	NCT02265276
GI262570	GlaxoSmithKline	HCV	II	NCT00244751
Angiotensin antagonists				
Losartan	Hospital Clinic of Barcelona	HCV; liver fibrosis	IV	NCT00298714/[57]
	Newcastle University	NASH	III	NCT01051219
	Miriam Vos, MD; National Institute of Diabetes and Digestive and Kidney Diseases; Children's Healthcare of Atlanta	NAFLD	II	NCT01913470
Irbesartan	Inserm-ANRS/Sanofi	HCV	III	NCT00265642
Candesartan	Kaiser Permanente	HCV	II	NCT00930995
	Yonsei University	ALD	I–II	NCT00990639
Moexipril (ACE inhibitor)	Mayo Clinic/UCB Pharma	PBC	II	NCT00588302
Cytokines/cytokines inhibitors				
Pirfenidone	University of Guadalajara	HCV	Pilot	[58]
INFγ	Huntington Medical Research Institutes	HBV	II	[59]
INFγ1b	InterMune	Liver fibrosis; cirrhosis	II	NCT00043303

Table 3a. Clinical trials with reduction of liver fibrosis as an end point. (From *Fagone P et al.* Drug Discov Today. 2015)

Biologics				
Simtuzumab (anti-LOXL2)	Gilead Sciences	Liver fibrosis; HCV	II	NCT01707472
	Gilead Sciences	Liver fibrosis	II	NCT01452308
	Gilead Sciences	NASH	II	NCT01672866
	Gilead Sciences	PSC	II	NCT01672853
FG-3019; (anti-CTGF)	FibroGen	HBV	II	NCT01217632
Mesenchymal stem cell (MSC) injection	Yonsei University	Alcoholic liver cirrhosis	II	NCT01741090
Autologous mesenchymal stem cell transplantation	University of Tehran	Liver cirrhosis	II	NCT00476060
Umbilical cord-MSC transplantation	Fourth Military Medical University	Liver cirrhosis; end-stage liver disease	I–II	NCT01728727
MSC	Chaitanya Hospital, Pune	Liver cirrhosis	I–II	NCT01877759
Autologous bone marrow MSC transplantation	Nanjing PLA General Hospital	Liver cirrhosis	III	NCT01854125
Anticoagulants				
Warfarin, ximelagatran	Imperial College London	Liver fibrosis	II	NCT00180674
Glucagon-like peptide-1 analog				
Liraglutide	Sun Yat-Sen University	NAFLD	II	[60]
Exenatide	Ruhr University of Bochum	NAFLD	IV	NCT01208649
	Indiana University	NAFLD	II–III	NCT00650546
Gal3 inhibitor				
GR-MD-02	Galectin Therapeutics	NAFLD	II	NCT02421094
		NASH	II	NCT02462967
FXR agonists				
Obeticholic acid (OCA)	Intercept Pharmaceuticals	Liver cirrhosis, biliary	III	NCT02308111
		PBC	III	NCT01473524
		PSC	II	NCT02177136
		NASH	III	NCT02548351
	National Institute of Diabetes and Digestive and Kidney Diseases	NAFLD; NASH	II	NCT01265498
CB1 inverse agonists				
Rimonabant (SR141716)	Sanofi	Fatty liver	III	NCT00577148; NCT00576667

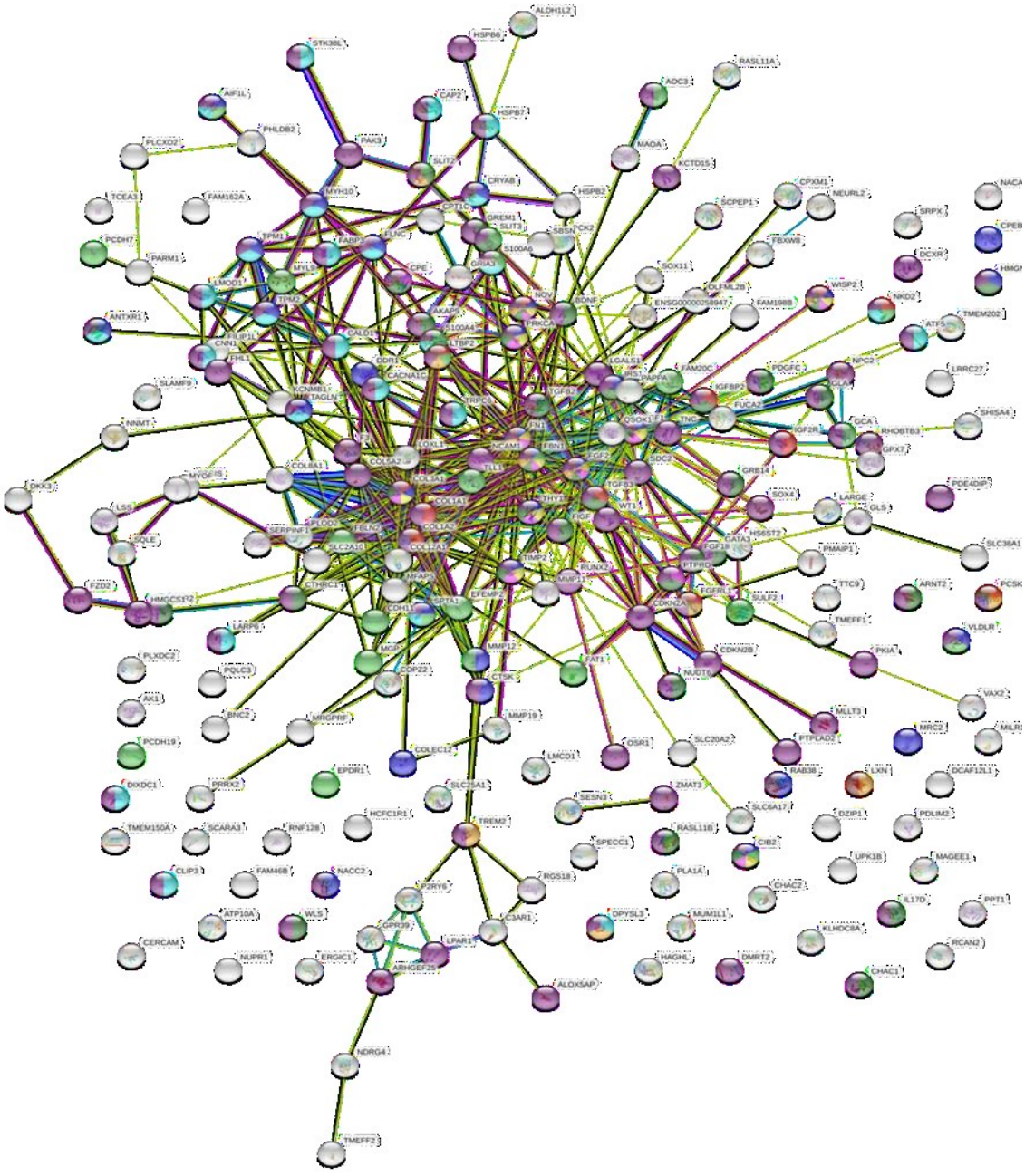
Table 3b. Clinical trials with reduction of liver fibrosis as an end point. (From *Fagone P et al.* Drug Discov Today. 2015).

Drug Prediction Analysis

The L1000CDS2 web-based utility was used to identify potential drugs for the treatment of liver fibrosis. L1000CDS2 searches among chemically perturbed gene expression profiles that match, in a opposite manner, the input gene signature. The L1000 transcriptomic database belongs to the Library of Integrated Network-based Cellular Signatures (LINCS) project, a NIH Common Fund program. When a gene list is submitted to L1000CDS2, the search engine compares the input gene list to the differentially expressed genes (DEGs) in the LINCS L1000 data and outputs the top 50 matched signatures. The score represents the overlap between the input genes and the signature DEGs divided by the effective input. L1000CDS2 comprises drug-perturbed gene expression profiles from 62 cell-lines and >3,900 small molecules.

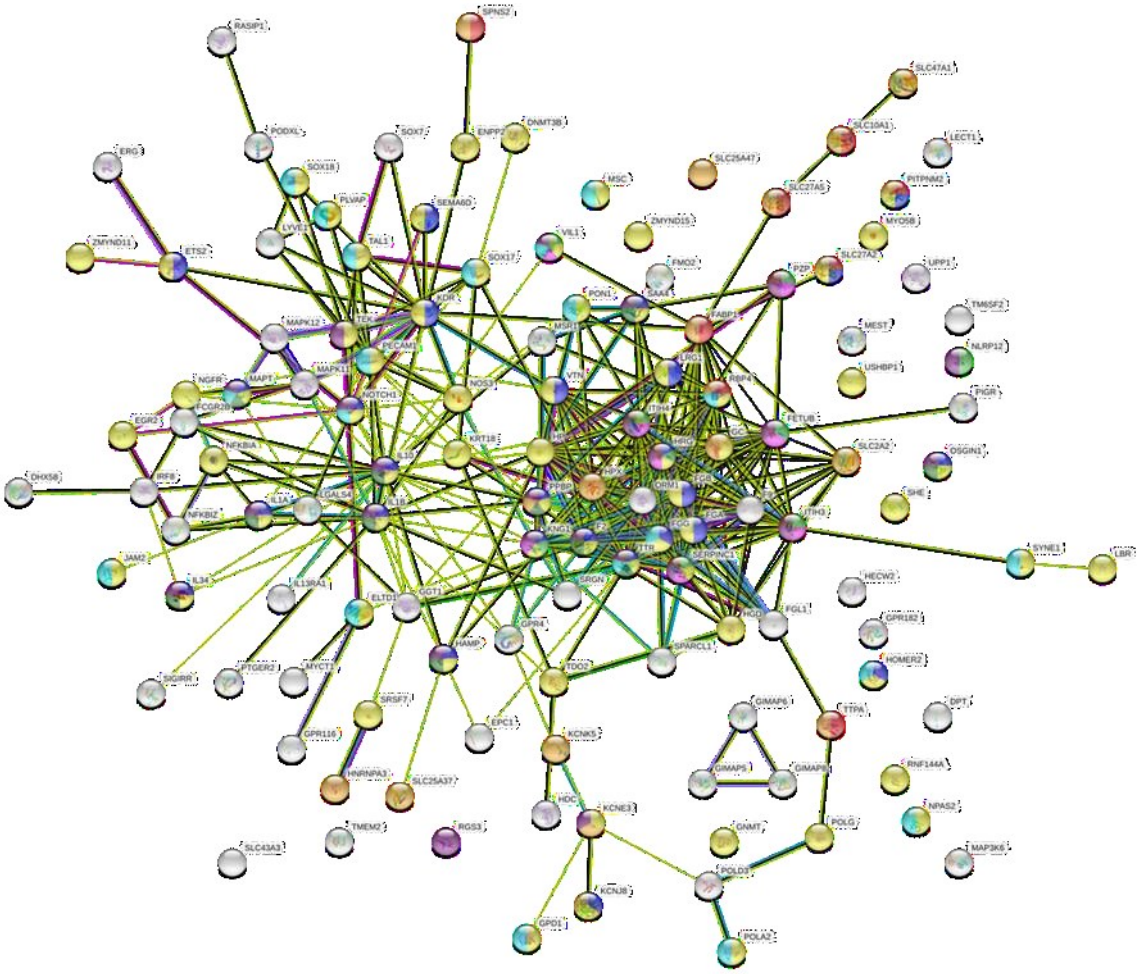
We have previously identified a pro-fibrogenetic gene signature by performing a meta-analysis of publicly available microarray datasets on hepatic stellate cell activation [62]. The gene signature consisted of 254 and 132 genes upregulated and downregulated, respectively. This gene signature was used as imput for the L1000CDS2 analysis.

A gene network, constructed with the upregulated genes of the fibrogenetic signature is present in *Figure 18*. A gene network constructed on the downregulated genes of the profibrogenetic pathway is represented in *Figure 19*. In both *Figure 18 and 19*, different colors have been assigned to each gene, based on their molecular function. The L1000CDS2 web-based utility was used to predict drugs targeting the liver fibrosis gene signature. *Figure 20* shows the Clustergram of the L1000 perturbations with the most anti-similar signatures to the input genes. The clustergrammer shows in colums the identified drugs and in rows the input genes (in red the upregulated and in blue the downregulated), and for each row-column combination indicates the genes which are inversely modulated by the drugs. Some of these drugs are already used in the clinic setting or are currently being tested in clinical trials.



Molecular Function (GO)			
GO-term	*description*	*count in gene set*	*false discovery rate*
GO:0019838	growth factor binding	12 of 126	3.18e-05
GO:0044877	protein-containing complex binding	31 of 968	5.55e-05
GO:0005509	calcium ion binding	26 of 700	5.55e-05
GO:0005178	integrin binding	11 of 122	5.55e-05
GO:0050839	cell adhesion molecule binding	13 of 200	0.00011
GO:0005102	signaling receptor binding	38 of 1513	0.00038
GO:0008092	cytoskeletal protein binding	26 of 882	0.00092
GO:0005539	glycosaminoglycan binding	12 of 219	0.00092
GO:0005515	protein binding	106 of 6605	0.00092
GO:0008201	heparin binding	10 of 161	0.0014

Figure 18. A gene network constructed with the *upregulated* genes of the fibrogenetic signature.



Molecular Function (GO)			
GO-term	*description*	*count in gene set*	*false discovery rate*
GO:0005319	lipid transporter activity	8 of 134	0.0017
GO:0005102	signaling receptor binding	26 of 1513	0.0017
GO:0061134	peptidase regulator activity	9 of 211	0.0019
GO:0005515	protein binding	65 of 6605	0.0022
GO:0004866	endopeptidase inhibitor activity	8 of 169	0.0022
GO:0048018	receptor ligand activity	11 of 458	0.0126
GO:0046983	protein dimerization activity	20 of 1301	0.0140
GO:0005215	transporter activity	19 of 1223	0.0157
GO:0098772	molecular function regulator	24 of 1793	0.0171
GO:0004867	serine-type endopeptidase inhibitor activity	5 of 94	0.0171

Figure 19. A gene network constructed on the *downregulated* genes of the profibrogenetic pathway.

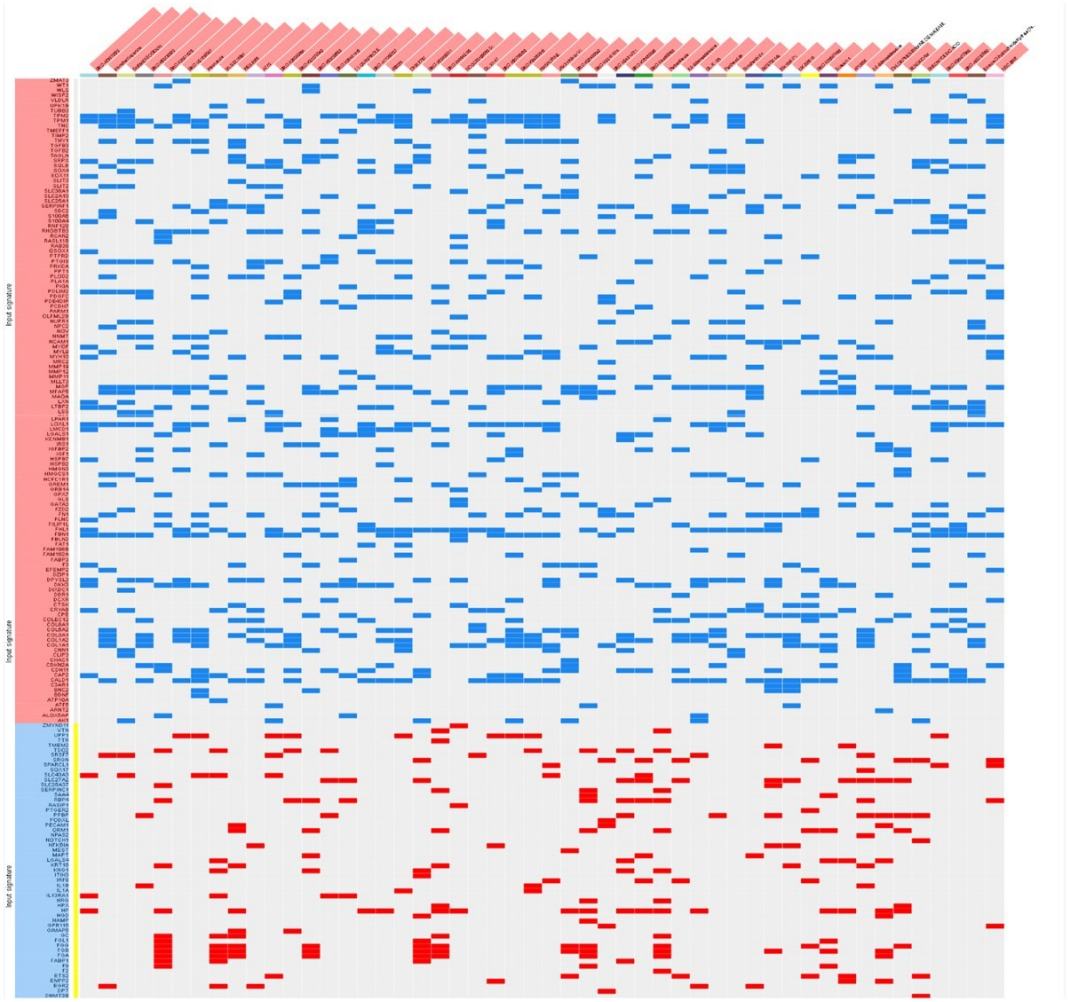


Figure 20. Clustergram of the L1000 perturbations with the most anti-similar signatures to the input genes. The clustergrammer shows in colums the identified drugs and in rows the input genes (in red the upregulated and in blue the downregulated), and for each row-column combination indicates the genes which are inversely modulated by the drugs.

The top 3 predicted small molecules resulted to be: sarmentogenin, a steroid lactone closely related to digitoxigenin, found in plants of the genus Strophanthus, obtained by hydrolysis of sarmentocymarin, and used in a synthesis of cortisone; homoharringtonine (a.k.a., Omacetaxine mepesuccinate), a natural plant alkaloid derived from Cephalotoxus fortune and approved by the US FDA in 2012 for the treatment of adult patients with CML with resistance and/or intolerance to two or more tyrosine kinase inhibitors; and BRD-A28970875 (puromycin), aminonucleoside antibiotic, derived from the Streptomyces alboniger bacterium (*Table 4*). Other notable small molecueles identified comprise : BRD-K60230970 (a.k.a., MG132), a potent, reversible, proteasome inhibitor;

importazole, an inhibitor of the transport receptor importin-β; BRD-K51290057, a potent synthetic retinoid withhigh affinity for RAR-α and RAR-β receptors and low affinity for cellular retinoic acid binding protein (CRABP).

The most promising drug combinations are represented by the association between : 1) sarmentogenin and BRD-K30511626 ; 2) BRD-A28970875 and BRD-K30511626 ; and 3) BRDK60230970 and BRD-K30511626 (*Table 5*).

Rank	Score	Perturbation	Cell-line	Dose	Time
1	0.0883	SARMENTOGENIN	HA1E	10.0um	24.0h
2	0.0883	Homoharringtonine	HA1E	10.0um	24.0h
3	0.0883	BRD-A28970875	SKB	10.0um	24.0h
4	0.0848	BRD-K60230970	ASC	10.0um	24.0h
5	0.0813	Importazole	HA1E	60.0um	24.0h
6	0.0813	BRD-K51290057	SKB	10.0um	24.0h
7	0.0813	BRD-K30511626	VCAP	10.0um	6.0h
8	0.0777	BRD-K52075040	HA1E	44.4um	24.0h
9	0.0777	BRD-K36055864	SKB	10.0um	24.0h
10	0.0777	AZ20	HA1E	10um	24h
11	0.0777	WEB 2086	VCAP	10.0um	24.0h
12	0.0777	MLS-0216981	HA1E	10.0um	6.0h
13	0.0742	CHR 2797	HA1E	80.0um	24.0h
14	0.0742	598226	SKB	10.0um	24.0h
15	0.0742	BRD-A17065207	SKB	10.0um	24.0h
16	0.0742	(-)-QUINPIROLE	HCC515	10.0um	24.0h
17	0.0742	BRD-K08451418	MCF7	10.0um	6.0h
18	0.0742	BRD-K93258693	A549	10.0um	24.0h
19	0.0707	JAK3 Inhibitor VI	HA1E	10.0um	24.0h
20	0.0707	AMSACRINE	HA1E	10.0um	24.0h
21	0.0707	BRD-K94325918	HA1E	10.0um	24.0h

Table 4. Showing the small molecules predicted to have an antifibrotic effect.

Rank	Score	Combination	
1	0.1625	1. SARMENTOGENIN	7. BRD-K30511626
2	0.159	3. BRD-A28970875	7. BRD-K30511626
3	0.159	4. BRD-K60230970	7. BRD-K30511626
4	0.159	4. BRD-K60230970	12. MLS-0216981
5	0.1555	2. Homoharringtonine	3. BRD-A28970875
6	0.1555	2. Homoharringtonine	7. BRD-K30511626
7	0.1555	7. BRD-K30511626	9. BRD-K36055864
8	0.1555	3. BRD-A28970875	10. AZ20
9	0.1555	3. BRD-A28970875	30. 2,5-dideoxyadenosine
10	0.1555	2. Homoharringtonine	31. Parbendazole
11	0.1555	2. Homoharringtonine	35. BRD-K55191674
12	0.1519	6. BRD-K51290057	7. BRD-K30511626
13	0.1519	1. SARMENTOGENIN	11. WEB 2086
14	0.1519	2. Homoharringtonine	12. MLS-0216981
15	0.1519	5. Importazole	12. MLS-0216981
16	0.1519	3. BRD-A28970875	18. BRD-K93258693
17	0.1519	4. BRD-K60230970	18. BRD-K93258693
18	0.1519	1. SARMENTOGENIN	25. BRD-K49945136
19	0.1519	2. Homoharringtonine	25. BRD-K49945136
20	0.1519	1. SARMENTOGENIN	35. BRD-K55191674
21	0.1519	1. SARMENTOGENIN	48. UK 356618

Table 5. The most promising drug combinations are represented by the association between : 1) sarmentogenin and BRD-K30511626 ; 2) BRD-A28970875 and BRD-K30511626 ; and 3) BRDK60230970 and BRD-K30511626

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.

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