Experimental in vitro and in vivo Efficacy Assessment of Safironil as an Antifibrotic Therapy Potentially Enhance Reversal of Liver Fibrosis with Praziquantel Combination in Murine Chronic Schistosomiasis Mansoni

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Abstract: Background: Liver fibrosis is the principle feature of injury caused by chronic liver disease leading to clinical death. Schistosomiasis causes pathology in 200 million individuals worldwide. The perisinusoidal stellate cells of the liver in an injury milieu undergo activation, acquiring a myofibroblast-like phenotype. In this state, they are the principle source of collagen and related proteins in fibrosis. Aim: The present studies evaluate in vitro, the mechanism of action of an antifibrotic compound Safironil, which was designed as competitive inhibitor of collagen protein synthesis. Results: In vivo, the studies investigate its possible effect on reversal of liver fibrosis by combination with Praziquantel in mice. In vitro, safironil prevented fibrocytoblast activation monitored by the level of collagen I mRNA or smooth muscle α-actin and fibrogenesis judged by collagen type I and III and laminin production. In animals, Safironil had no effect on liver granulomas sizes without altering total hydroxyproline but alter the pattern of fibrosis by increasing type III and decreasing type I collagen deposition. When Safironil was combined with Praziquantel, best results were attained. The mechanism is probably through stop of new liver insult and interruption of collagen type I synthesis with attenuation of pre-existent collagen. Conclusion: Although, these results are promising in experimental schistosomiasis, further studies are required to study its possible application in humans.

Key words: Schistosoma mansoni, mice, liver fibrosis, anti-fibrotic, praziquantel

INTRODUCTION

Hepatic fibrosis is a common response to various insults such as parasitic infection, chronic viral infection and autoimmune diseases, hereditary and toxic damage (Wang et al., 1998). Hepatic fibrosis is associated with activation of Hepatic Satellite Cells (HSC), the major source of Extracellular Matrix (ECM) proteins. The predominant ECM protein synthesized by the HSC is collagen type I. The progressive accumulation of connective tissue results in destruction of normal tissue architecture and function (Bruck et al., 2001). In schistosomiasis, healing of parasite egg-induced liver granuloma can lead to periportal fibrosis and may lead to obstruction of portal flow with subsequent portal hypertension and bleeding esophageal varices (Dunn and Kamel, 1981). Fibrogenesis is a dynamic potentially reversible process mediated through the immunological responses to parasite eggs trapped in the liver (Wynn et al., 1995). It is initiated and maintained by macrophages, lymphocytes and myofibroblast with certain growth factors as transforming growth factor β (TGFβ), and fibrogenic mediators (Wyles, 1992). Both CD4+ lymphocytes and Kupffer cells produce TGFβ, after contact with soluble egg antigen (SEA). TGFβ, stimulates HSC activation and production of matrix proteins (Kresina et al., 1994). In Schistosomiasis, collagen synthesis is quantitatively prominent specially type I, after its cross linking and it has been a target for therapeutic strategies against fibrosis (Bissel and Mahe, 1996). A continuous murine cell line (GRX) was obtained from fibrotic granulomas in C3/HaN mice liver by experimental infection with Schistosoma mansoni (Borojevic et al., 1985). These cells are considered to be representative of liver connective tissue cells, responsible for fibroplasia in liver fibrotic and granulomatous reactions. They also display simultaneous characteristics of myofibroblasts and HSC. This GRX cell line excretes ECM molecules such as collagen I, collagen III and fibronectin (Guo and Friedmann, 2007). GRX cells plated on culture plastic, during the initial 3 to 5 days ex vivo acquires myofibroblast-like characteristics. Because this change mimics many aspects of activation in vivo, it is regarded as a culture model of the wound healing response (Friedman, 1993). HOE 077 (pyridine-2, 4-dicarboxylic-di (2-methoxyethyl) amide) and its congeners Safironil (SAF) represent a new class of anti-collagen compounds, designed as competitive inhibitor of propyl-4-hydroxylase (Proeckop and Kivirikko, 1995). The latter enzyme is essential for the formation of a stable collagen.
trimmer (Hanauske-Abel, 1996). All general inhibitors of collagen however, have potential for compromising the integrity of collagen-rich tissues such as skin, bone and vasculature. Safronil is an inactive pro-drug by amidation of its carboxyl group. Its conversion to the active form requires oxidative deamination, a function performed by the cytochrome P-450 family of heme proteins (Hanauske-Abel and Gunzler, 1982). This requirement was expected to confer a considerable degree of liver specificity, in that the concentration of cytochrome P-450 is far greater in liver than in other tissues. In experimental work HOE 077 was shown to block production of collagen by hepatocytes in culture (Clement et al., 1991). It also blocked fibrosis in female rats treated with carbon tetrachloride (CCL), with no measurable effects on normal tissues (Buckel et al., 1991). The goal of this study was to assess the in vitro effect of Safronil on the activation of fibrotic granulomas cells (GRX), its differentiation to myofibroblast and its ability of ECM proteins synthesis and to investigate the possible antifibrotic role of Safronil on experimentally induced chronic schistosomal hepatic fibrosis in mice.

MATERIALS AND METHODS

Cell line and culture: C3HeB/FeJ mice continuous cell line (GRX), was generously provided by Dr. Borjovic (Instituto de Quimica, Rio de Janeiro, Brazil). Cultures were maintained in the Dulbecco's Modified Essential Medium (DMEM) with 10% fetal bovine serum, 3 μg mL⁻¹ of HEPES buffer, 1% Penicillin and 1% Streptomycin. Cells were cultured at a concentration of 2×10⁵ cells/well in a total volume of 3 mL using a Lab-Tek tissue culture chambers slides (Miles, Naperville, IL). Cells were stimulated in vitro for morphological phenotype transformation and matrix proteins excretion by adding TGFβ, MAb at 0.1 ng mL⁻¹. Its salient features include de novo production of smooth muscle α-actin as well as markedly increased expression of collagen I.

In vitro analytical procedures: Total RNA was extracted from the cultured cells using TRIzol reagent according to the manufacturer's instructions (Gibco BRL, Life Technologies, Rockville, MD). RNase protection assay was performed using RiboQuant™ Multi-probe RNase Protection Assay System with the mCK-1 including mouse collagen (α-1) type I, multioptera template sets (Pharmingen, San Diego, CA). Radioactivity was quantified by 32P imaging on a Storm-680 scanner and data analyzed using Image Quant™ Version 1.11 (Molecular Dynamics, Sunnyvale, CA). The smooth muscle isoform of α-actin was measured by quantitative Western blot of extracts from cultured cells, as described by Rockey et al. (1992) and equal amounts of protein extract were loaded. Studies with cultures were replicated. Synthesis of collagen was assayed as incorporation of [3H] proline into protein digestible by highly purified collagenase, as described by Maher et al. (1994).

Animals and Schistosoma mansoni parasite infection: Adult female, Swiss albino mice, 4-5 weeks old, weighing 16-18 g each, were purchased from the Schistosome Biological Supply Program (SBSP), Theodor Bilharz Research Institute (TBRI), Egypt. Approval of the institutional animal ethical committee (protocol # 1096-5), was obtained for the animal studies and Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines were followed throughout the experiment. Eighty mice were submitted to subcutaneous injections with a dose of 80 Schistosoma mansoni cercariae/ mouse, as described by (Peters and Warren, 1969).

Application of antifibrotic chemotherapy, Safronil (SAP) with and without antiparasitic chemotherapy, Praziqantel (PZQ): SAP was supplied in vials and stored at 4°C from (Hoechst Company, Aktiengesellschaft and Frankfurt, Germany). It was administered by gavage or provided in drinking water at a concentration of 1.5 mg mL⁻¹, from the 3rd month post infection (p.i.) till the 6th month p.i. Control groups were given normal saline. PZQ was available as (Biltricide) 600 mg tablets from (Bayer Company). It was emulsified with 30% glycerol prior to its use and given at 12 week p.i, in a dose of 500 mg kg⁻¹/dose according to Islam et al. (1996). The dose was followed by another dose after 9 days to assure killing of all eggs in the tissue since PZQ has the capacity to kill only mature ova according to Giboda and Smith (1994).

Mice were divided into 5 groups of 20 mice each. Group 1 mice served as +ve control (infected non treated). Group 2 was received PZQ (infected and PZQ treated). Group 3 was received SAF and PZQ (infected, PZQ and SAF treated). Group 4 was received SAF alone (infected and SAF treated). Group 5 was -ve control (non infected, non treated). Ten mice of groups I, II, III, IV were sacrificed at the end of 20th week p.i. The remaining numbers from all groups were sacrificed at the end of 24th week p.i. After scarification the liver of each mouse was taken, weighed and divided into 3 portions; one part was used for tissue digestion, the second was preserved in 10% formalin for histopathological examination and the third part was kept frozen at -70°C for hydroxyproline assay and collagen determination.
Parasitological and Biochemical assays: For egg counting in the liver, fragments of liver tissue were weighed and left in 0.5% potassium hydroxide solution for digestion and counting of *S. mansoni* eggs according to (Cheever, 1988). The results were expressed as the number of eggs/g liver tissue. Collagen formation was estimated by total proline and hydroxyproline content of each individual liver using L-azetidine-2-carboxylic acid as an internal standard. After derivatization with 4-chloro-7-nitrobenzofurazan, the components were analyzed using Programmable Solvent module 126 columns, 4.6×75 mm and a Gynotek Fluorescence detector FR-530 as described by (Palmerini *et al.*, 1985).

Histopathological and immunofluorescence staining studies: Liver biopsies were fixed in 10% formalin, dehydrated, embedded in paraffin. Sections 4 μm-thicknesses were stained with haematoxylin and eosin (H and E) for histopathological examination. A measurement of the size of the granulomas was done by the use of ocular micrometer lens fitted on a light microscope. The mean diameter of each Granuloma was obtained by measuring two perpendicular diameters. For each section, ten granulomas were measured and the mean diameter of all lesions then calculated using the method described by Jacobs *et al.* (1997). Semi-quantitation of collagen isotypes I and III was carried out according to Gabr *et al.* (1995). Briefly, the frozen liver sections were stained with the corresponding FITC conjugated MAb for 45 min at 4°C, then the intensity of the fluorescence was measured by an automated photometry connected to a ultra-violet microscope.

Statistical analysis: It was done by using SPSS, Version 8.0 software. ANOVA test was used to test significance between different treatments at p = 0.05.

RESULTS

*In vitro studies*: Before testing the effect of Saffronil on cell culture activation, toxicity studies were performed. No toxic effect was seen at concentrations up to 2 mg mL⁻¹, as judged by cell detachment from the substratum, trypan blue staining, or leakage of cellular lactate dehydrogenase to the medium. The antifibrogenic effect of Saffronil was examined and showed that it reduced expression of mRNA for type I collagen in a dose dependent manner (Fig. 1). To exclude the possibility that Saffronil was acting selectively on collagen I expression, an independent parameter of cell culture activation, smooth muscle α-actin (which is a good marker for myofibroblast activation) was also monitored. Saffronil reduced the expression of this protein (Fig. 2). The data provide direct evidence that the antifibrogenic effect of Saffronil involves down-regulation

![](image1.png)

**Fig. 1**: Collagen I mRNA in GRX cells in primary culture. The indicated concentrations of Saffronil were added on day 2 and their effect was evaluated by RNase protection assay. The data are expressed relative to control cells, set at 100% (n = 4)

![](image2.png)

**Fig. 2**: Expression of smooth muscle alpha-actin by GRX cells culture with Saffronil. After 5 days culture, protein extracts were prepared and analyzed by qualitative immuno blot

![](image3.png)

**Fig. 3**: Effect of Saffronil on matrix proteins production of primary cell culture after 5 days activation of cell culture activation. Saffronil increased the production of type III collagen and decreased the production of type I collagen by the primary cultured myofibroblast cells (Fig. 3).
Table 1: The weight of liver, number of egg/g liver and the size of granuloma among the different groups (Mean±SD)

<table>
<thead>
<tr>
<th>Number of Mice</th>
<th>Weight of liver (g) (Mean±SD)</th>
<th>Egg count/g liver tissue (Mean±SD)</th>
<th>Size of granuloma (µM) (Mean±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20th wk</td>
<td>24th wk</td>
<td>20th wk</td>
</tr>
<tr>
<td>1</td>
<td>10</td>
<td>7</td>
<td>1.9±0.4</td>
</tr>
<tr>
<td>2</td>
<td>9</td>
<td>8</td>
<td>1.4±0.9*</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>7</td>
<td>1.27±0.1*</td>
</tr>
<tr>
<td>4</td>
<td>9</td>
<td>7</td>
<td>1.7±0.3</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>-</td>
<td>1.2±0.14</td>
</tr>
</tbody>
</table>

*Significant compared to groups (1 and 4). **Significant compared to groups (1, 2 and 4)

Table 2: Estimation of hydroxyproline and total collagen contents of livers in the studied groups

<table>
<thead>
<tr>
<th>Liver hydroxyproline (mg g⁻¹ wet liver weight)</th>
<th>Calculated collagen (mg g⁻¹ wet liver weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20th wk</td>
<td>24th wk</td>
</tr>
<tr>
<td>1</td>
<td>0.51±0.21</td>
</tr>
<tr>
<td>2</td>
<td>0.48±0.18*</td>
</tr>
<tr>
<td>3</td>
<td>0.44±0.15*</td>
</tr>
<tr>
<td>4</td>
<td>0.46±0.2**</td>
</tr>
</tbody>
</table>

*Significant in relation to groups (1 and 4). **Significant in relation to group (1) (p<0.05)

**In vivo studies:** The results of parasitological assay of antifibrotic efficacy of Safironil including liver weight and egg count/g liver tissue were presented in Table 1. The results of biochemical assay of hepatic hydroxyproline and collagen contents were presented in Table 2.

**DISCUSSION**

Prolonged liver injury results in hepatocytes damage, which triggers activation HSC and recruitment of inflammatory cells into the liver. The HSC play a critical role in fibrogenesis. They produce collagen type I and secrete pro-fibrogenic cytokines and inhibitors of matrix-degrading enzymes (tissue inhibitor of matrix metalloproteinase) (MMP), causing the production of extracellular matrix deposition over degradation. Hepatic fibrosis was historically thought to be a passive and irreversible process. However, many clinical and experimental studies suggest that this process can be reversed, including the apoptosis of activated HSC. Thus, HSC represent an appealing target for antifibrotic therapy (Kisseleva and Brenner, 2006). Many lines of therapy were tried to reduce liver collagen formation, however, none was satisfactory. Steroids were used as inhibitors to prolyl hydroxylase and anti-inflammatory (Guzelian et al., 1984). Praziqantel has been used as an antifibrotic drug by eliminating antigen source, but it interrupts immune modulation, hence causing poor scare absorption in the liver (Giboda and Smith, 1994). Colchicine is a microtubular disruptive drug but owing to its side effects, its use as a long term therapy was not widely approved (Budawy et al., 1996). Halofuginone, the synthetic compound of a natural product Dicrhoa febrifuga Louir, is used as an antifibrotic agent in rat hepatic stellate cells (Gnansky et al., 2003). However, Halofuginone worsened liver fibrosis in bile duct obstructed rats (Van de Casteele et al., 2004).

In this study, the mechanism of action of Safironil is shown to involve largely, if not exclusively, HSC activation. The specificity of Safironil for the liver in the chronic Schistosomiasis mansoni was believed to reflect a requirement for conversion of the pro-drug to an active inhibitor, a process in which the liver is pre-eminent because of its high concentration of cytochrome P-450. In retrospect, it appears, rather, that the effect of Safironil is targeted to the myofibroblast responses to wound repair. The combination of anti schistosomal drug and safironil led to the most profound reduction in liver granuloma size; while in immune fluorescence section, there was resolution of collagen fibers in mice treated and sacrificed late at the 24th wk. These results agreed with Giboda and Smith (1997).

**CONCLUSION**

In summary, we have demonstrated that the novel compound Safironil reduces fibrogenesis in liver insult by S. mansoni egg deposition by inhibiting stellate cell activation. As such it target inflammation and wound repair and mitigate concern for the potential side effects of direct inhibitors of collagen protein synthesis. This raises the interesting possibility that a compound such as Safironil may target wound repair beyond the liver and has a therapeutic potential for a variety of fibrosing diseases.

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REFERENCES


