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Research Article

Reversal of Liver Fibrosis in Chronic Murine *Schistosomiasis mansoni* by Safironil/Praziquantel

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Abstract

Purpose: *To evaluate the safety, pharmacological effect and mechanism of action of an antifibrotic compound, safironil (SAF)/praziquantel (PZQ) combination on reversal of liver fibrogenesis in chronic murine Schistosomiasis mansoni.*

Method*s: The antifibrotic effect of 0.5, 1 and 2 mg/ml of SAF was evaluated in vitro myofibroblast cell culture, using RNAse protection assay for collagen I mRNA expression and quantitative immunoblot for smooth muscle α-actin protein extract. Ninety Swiss albino mice were infected with 50 Schistosoma mansoni cercariae. SAF was provided in drinking water at a concentration of 1.5 mg/ml while praziquantel (PZQ) was given by gavage in a dose of 500 mg/kg. Mice, divided into five groups: infected non-treated; infected and PZQ-treated; infected PZQ- and SAF-treated; infected and SAF-treated; and control. After sacrificing the animals, the liver of each mouse was taken, weighed and used for histopathological examination, hydroxyproline assay and collagen determination.*

Results*: SAF prevented myofibroblast activation at the pre-transcriptional level in a dose-dependent manner as monitored by collagen I mRNA levels (expression reduced by 40, 70 and 90 % at doses of 0.5, 1 and 2 mg/ml, respectively) or by smooth muscle α-actin (expression reduced by 70, 85 and 95 %, respectively). SAF decreased the production of collagen I by 60 % and laminin by 55 % but increased collagen III by 50 % relative to control. SAF had no effect on liver granuloma size and did not alter total hydroxyproline but altered the pattern of fibrosis by increasing collagen III and decreasing collagen I deposition. The most significant reduction in liver fibrosis was noticed in mice treated with SAF combined with PZQ. No toxic pharmacological effect was noticed during SAF treatment.*

Conclusion*: When SAF was combined with PZQ, augmented reduction of liver fibrogenesis was achieved. The mechanism is probably through inhibition of new liver injury induced by parasite egg deposition and interruption of collagen type I synthesis with attenuation of pre-existent collagen..*

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Keywords: *Schistosomiasis mansoni, Mice, Liver fibrosis, Antifibrotic, Praziquantel*

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INTRODUCTION

Hepatic fibrosis is a common response to various insults to the liver such as parasitic infection, chronic viral infection, autoimmune diseases, and hereditary and toxic damage [1]. Hepatic stellate cells (HSC) plays a crucial role during liver fibrosis. After hepatocyte damage, the cell becomes activated and starts to proliferate, during which it transforms from a resting vitamin A-storing cell into an activated myofibroblast (MF)-like cell. Activated HSCs produce excessive amounts of extracellular matrix (ECM) compounds and inhibitors of matrix degredation. Therefore, this cell type is a key player in the fibrogenic response that may eventually lead to liver cirrhosis with subsequent hepatic fibrosis [2].

In schistosomiasis, healing of parasite egg-induced liver granuloma can lead to periportal fibrosis, and bleeding esophageal varices. Fibrogenesis is a dynamic potentially reversible process mediated through immunological responses to parasite eggs trapped in the liver… Collagen synthesis (especially type I) is quantitatively prominent and has been a target for therapeutic strategies against fibrosis [3]. A continuous murine cell line (GRX), has been obtained from fibrotic granulomas in mice liver experimentally infected with *S. mansoni* [4]. These cells display simultaneous characteristics of myofibroblasts and HSC. GRX cell line excretes ECM molecules such as collagen I, collagen III and fibronectin. Thus, it is regarded as a culture model of the wound healing response [5].

HOE 077 [pyridine-2, 4-dicarboxylic-di (2-methoxyethyl) amide] and its congener, safironil (SAF), represent a new class of anti-collagen compounds designed as competitive inhibitor of propyl-4-hydroxylase [6]. This enzyme is essential for the formation of a stable collagen trimmer [7]. All general inhibitors of collagen, however, have potential for compromising the integrity of collagen-rich tissues such as skin, bone, and vasculature. SAF is an inactive pro-drug obtained by amidation of its carboxyl group. Its conversion to the active form requires oxidative deamidation, a function performed by the cytochrome P-450 family of heme proteins. This requirement confers a considerable degree of liver specificity, in that the concentration of cytochrome P-450 is far greater in liver than in other tissues. It has been reported that HOE 077 blocks production of collagen by hepatocytes in culture [8].

The goal of this study was to assess the pharmacological effect of SAF on the activation of GRX and its production of ECM compounds in cell culture, as well as to investigate the pharmacological effect of SAF augmented with PZQ on reversal of liver fibrogenesis in mice with chronic *Schistosomiasis mansoni*.

EXPERIMENTAL

Cell line and culture

C3/HeN mice continuous cell line (GRX) was generously provided by Dr(Borojevic R), Instituto de Quimica, Rio de Janeiro, Brazil. Cultures were maintained in Dulbecco's Modified Essential Medium with 10% fetal bovine serum, 3 µg/ml of HEPES buffer, 1% penicillin and streptomycin. Cells were cultured at a concentration of 2 x 10^5 cells/well in a total volume of 3 ml using Lab-Tek tissue culture chambers slides (Miles, Naperville, IL). The cells were stimulated *in vitro* for morphological phenotype transformation and matrix proteins excretion by adding transforming growth factor β_1 ($TGFB_1$) monoclonal antibody (MAb), at 0.1 ng/ml. 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 and toxicity studies

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, multiprobe template sets (Pharmingen, San Diego, CA). Radioactivity was quantified by ^{32}P 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 [9] and equal amounts of protein extract were loaded. Studies with cultures were replicated with at least three cell preparations. Synthesis of collagen was assayed as incorporation of [^3H] proline into protein digestible by highly purified collagenase, as described by [10]. Before testing the effect of SAF on GRX cell activation in culture, toxicity studies were performed judged by cell detachment from the substratum after heparinization followed by iodine radioactive scintillation cell counting, trypan blue staining for cell viability or cellular lactate dehydrogenase to the medium.

Animals and parasite Infection

Six-week-old female, Swiss albino mice, weighing 16 - 18 g each, were purchased from Theodor Bilharz Research Institute TBRI, Egypt. Parasite infection was done through subcutaneous injection of 50 *S. mansoni* cercariae into each mouse. Approval of the institutional ethical committee for animal studies was obtained and the sudy followed the Office of Environmental Health and Radiation Safety, ACUC Protocol # 1096-5. The animals were maintained according to accepted standards of animal care. The animals were housed in polypropline cages at 25 ± 2 ^oC and fed with standard pellet diet and water [11].

Treatment of animals

SAF was obtained in vials from Hoechst Company, Aktiengesellschaft, Frankfurt, Germany, and stored at 4^oC. It was administered by gavage in drinking water at a concentration of 1.5 mg/ml from the 3rd to the 6th month post-infection (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. at a dose of 500 mg/kg/dose. The dose was followed by another dose 9 days later to ensure the death of all eggs in the tissue. The mice were divided into 5 groups of 20 mice each. Group 1 mice served as positive control (infected, non-treated). Group 2 received PZQ (infected and PZQ-treated). Group 3 received SAF and PZQ (infected, PZQ and SAF-treated). Group 4 received SAF alone (infected and SAF-treated). Group 5 was negative control (non-infected, non-treated). Ten mice from Groups I, II, III and IV were sacrificed at the 20th week p.i. The remaining mice from all the groups were sacrificed at the 24th week p.i. After sacrifice, 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 % formol saline for histopathological examination while the third portion was kept frozen at -70 ^oC for hydroxyproline assay and collagen determination.

Parasitological, biochemical and liver function 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 the method of Cheever [12]. Briefly, 4 g of liver was shaking vigorously in a graduated flask with 0.1 KOH solution, shaken vigorously, allowed to stand for 24 h, shaken again, and 0.15 ml of the specimen placed on a glass slide for egg count. The results are 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. Following derivatization with 4-chloro-7-nitrobenzofurazan, the components were analyzed using Programmable Solvent Module 126 columns, 4.6 x 75 mm and Gynkotek fluorescence detector FR-530 as described by Palmerini et al [13]. Before animal sacrifice, heparinized blood was collected by heart-puncture and plasma levels for analine aminotrasferase (ALT) and aspartate aminotransferase (AST) were measured using mice kits, according to routine clinical chemical methods (Department of Clinical Chemistry, Maternity and Children Hospital, Madina Munawarah, KSA)

Histopathological and immunofluorescence staining studies

Liver biopsy specimens were fixed in 10 % formol saline, dehydrated and embedded in paraffin. Sections (4 µm thick) were stained with haematoxylin and eosin for histopathological examination. Measurement of granuloma size was carried out using a light microscope fitted with an ocular micrometer lens . 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* [14]. Semi-quantitation of collagen isotypes I and III was carried out as described by Gabr *et al* [15]. Briefly, the frozen liver sections were stained with the corresponding FITC conjugated MAb for 45 min at 4 ^{o}C and the intensity of the fluorescence measured by an automated photometer (Model FPH 64).

Statistical analysis

This was carried out using SPSS, version 8.0 software. Statistical significance was determined using one-way analysis of variance (ANOVA) and Student t-test. $P < 0.05$ was set as the level of significance.

RESULTS

In vitro data

No toxic effect of SAF was seen on cell culture at concentrations up to 2 mg/ml, as judged by cell detachment from the substratum, trypan blue staining, or leakage of cellular lactate dehydrogenase to the medium. SAF reduced expression of mRNA for type I collagen in a dose-dependent manner by 40, 70 and 90 % at doses of 0.5, 1 and 2 mg/ml, respectively (Table 1). To exclude the possibility that SAF 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. SAF reduced the expression of this protein in a dose-dependent manner by 70, 85 and 97 % at doses of 0.5, 1 and 2 mg/ml, respectively (Table 2). SAF increased the production of type III collagen and decreased the

Table 1: GRX primary cell culture collagen I mRNA evaluated by RNAse protection assay

SAF (mg/ml) cell culture on day 2	*Relative change (% of control)
0.5	60
1	30
2	10

**Data expressed relative to control cells, set at 100 % (n = 4, p > 0.05)*

Table 2: GRX primary cell culture expression of smooth α-actin prepared and analyzed by quatitative immunoblot

SAF (mg/ml) cell culture on day 5	*Relative change (% of control)
0	100
0.5	30
1	15
2	5

**data expressed relative to control cell, set at 100% (n =4), p > 0.05*

production of type I collagen by the primary cultured myofibroblast cells (Fig 1).

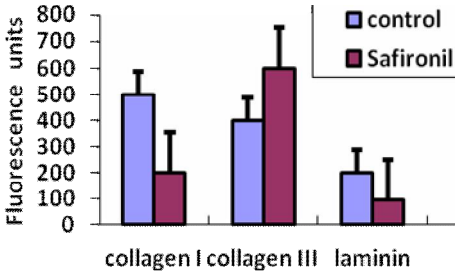

Figure 1: Effect of Safironil on matrix protein production of primary cell culture after 5 days activation

In vivo data

The results of parasitological assay of antifibrotic efficacy of safironil including liver weight and egg count/g liver tissue are presented in Table 3. The results of biochemical assay of hepatic hydroxyproline and collagen contents are shown in Table 4. The safety of safironil animal treatment on hepatic injury and hepatocytes damage was assessed by measuring serum levels of ALT and AST. No significant difference in serum levels of ALT was noticed between treated Groups, but AST level was higher in PZQ-treated mice compared to other Groups as shown in Table 5.

DISCUSSION

Prolonged liver injury results in hepatocyte damage, which triggers HSC activation and recruitment of inflammatory cells into the liver. 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 [16]. Hepatic fibrosis is historically thought to be a passive and irreversible process. However, results from many studies suggest that this process can be reversed, including the apoptosis of activated HSC. Thus, HSC is a ready target for antifibrotic therapy [17].

Table 3: Liver weight, number of eggs/g liver and granuloma size of the mice (mean ± SD)

Group	Number of mice		Weight of liver (g) (mean ± SD)		Egg count/g liver tissue (mean ± SD)		Size of granuloma (µm) (mean ± SD)	
	Week 20	*Week 24*	*Week 20*	*Week 24*	*Week 20*	*Week 24*	*Week 20*	*Week 24*
1	10	7	1.9 ± 0.4	1.8 ± 0.3	3250± 413	3365 ± 356	642.9 ± 26.5	516.6 ± 17.1
2	9	8	1.4± 0.9*	1.3± 0.2*	352± 270*	249 ± 240*	470.5 ± 25.1*	390.7 ± 19.1*
3	10	7	1.3± 0.1*	1.2± 0.1*	333± 260*	210 ± 280*	391.2 ± 21.8**	220.6 ± 18.7**
4	9	7	1.7 ± 0.3	1.6 ± 0.2	2951± 391	2918 ± 376	567.4 ± 26.5	412.3 ± 17.1
5	10	-	1.2 ± 0.1	-	-	-	-	-

**Significantly different compared to Groups 1 and 4 (p ≤ 0.05); **significant different compared to Groups 1, 2 and 4 (p ≤ 0.05)*

Table 4: Hydroxyproline and total collagen contents of mice liver (mean ± SD)

Group	Liver hydroxyproline (mg/g wet weight)		Calculated collagen (mg/g wetweight)	
	Week 20	*Week 24*	*Week 20*	*Week 24*
1	0.51 ± 0.21	0.66 ±0.19	4.1 ± 1.75	5.2 ± 1.39
2	0.48 ±0.18*	0.61 ± 0.14*	2.58 ± 1.29*	3.1 ±1.23*
3	0.44±0.13*	0.51 ± 0.13*	2.42 ±0.9*	1.89 ±0.8*1
4	0.46 ± 0.2**	0.59 ±0.18**	3.51 ± 1.43**	2.61 ±1.41**
5	0.21 ± 0.1	0.21 ± 0.1	1.3 ± 0.82	1.3 ± 0.82

**Significantly different in relation to Groups 1 and 4 (p ≤ 0.05) **Significantly different in relation to Group 1 (p ≤ 0.05)*

Table 5: Effect of drugs animals treatment on hepatocyte markers ALT and AST

Group	ALT (U/L)	AST (U/L)
1	90.5 ± 8.8	200.8 ± 45.8
2	116.8 ±10.5	314.5 ± 33.1*
3	107.8 ± 9.7	294.4 ± 41.2
4	99.8 ± 9.5	226.6 ± 34.9
5	40 ± 0.2	35.1 ± 0.1

**Significantly different compared to other treated Groups (p < 0.05)*

Several lines of therapy have been directed at the reduction of liver collagen formation. However, none has yielded satisfactory results [18].

Steroids have been used as inhibitors of prolyl hydroxylase and anti-inflammatory agents [19] while praziquantel has been used as an antifibrotic drug. It acts by eliminating antigen source, but interrupts immune modulation, thereby causing poor scar absorption in the liver [20]. Colchicine is a microtubular disruptive drug but owing to its side effects, its use as a long term therapy has not been widely approved [21]. Halofuginone, the synthetic compound of a natural product, *dichroa febrifuga* Lour, has been evaluated as an antifibrotic agent in rat hepatic stellate cells [22]. However, it has been reported that the compound worsened liver fibrosis in bile duct-obstructed rats [23].

The animals studies revealed that fibrosis process is reversible. Liver can undergo regression of fibrosis after withdrawal of the damaging stimulus, even in an advanced stage of cirrhosis [24]. In our studies, before testing the effect of safironil on GRX cell culture activation, toxicity studies were performed. No toxic effect of SAF was seen at concentrations up to 2 mg/ml. These findings are similar to those reported previously for hepatocytes in primary culture [8]. Our studies provide direct evidence that the antifibrinogenic effect of SAF involves down-regulation of cell culture activation. The specificity of safironil for the liver in chronic *Schistosomiasis mansoni* infection is believed to reflect a requirement for conversion of the pro-drug to an active inhibitor, a process in which the liver plays a pre-eminent role because of its high concentration of cytochrome P-450. In retrospect, it appearsthat the effect of safironil is targeted at 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 specimens, there was resolution of collagen fibers in treated mice that were sacrificed, i.e., at the end of 24 weeks.

CONCLUSION

We have demonstrated that the safironil reduces fibrogenesis in liver insult caused by *S. mansoni* egg deposition through inhibition of HSC activation. As such, it targets inflammation and wound repair and mitigates 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 fibrotic diseases

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