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Two Novel Antifibrotics, HOE 077 and Safironil, Modulate Stellate Cell Activation in Rat Liver Injury

Differential Effects in Males and Females

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The perisinusoidal stellate cells of the liver in an injury milieu undergo activation, acquiring a myofibroblast-like phenotype. In this state, they are the principal source of collagen and related proteins in fibrosis. The present studies evaluate the mechanism of action of two novel antifibrotic compounds, HOE 077 and Safironil, which were designed as competitive inhibitors of collagen protein synthesis. Fibrosis was induced in rats by administration of carbon tetrachloride, and activation was monitored as the level of collagen I mRNA or smooth muscle α-actin. Both male and female rats were studied. Stellate cell activation, rather than collagen synthesis, proved to be the target of both HOE 077 and Safironil in the intact liver. In culture, the drugs not only prevented the activation of stellate cells but also accelerated their deactivation. They were no more effective in co-cultures containing hepatocytes than in pure stellate cell cultures, indicating that metabolic conversion of HOE 077 was not required. Interestingly, the response of cells from females was greater than that of male cells, leading to the conclusion that stellate activation is sexually dimorphic. This finding may be relevant to the observation that fibrosis in chronic viral hepatitis progresses less rapidly and that hepatocellular carcinoma is less frequent in females than in males. ***(Am J Pathol 1998, 152:279–287)***

Fibrogenesis is integral to the wound-healing response. In chronic liver injury, however, it may progress to bridging fibrosis or cirrhosis, with disruption of lobular structure, altered blood flow, and impaired cellular function. Because collagen synthesis is quantitatively prominent in wound repair[1] and its pathway is understood in detail, it has been a focus of therapeutic strategies directed at fibrosis.[2] HOE 077 (pyridine-2,4-dicarboxylic-di(2-methoxyethyl)amide) and its congener Safironil represent a new class of anti-collagen compounds, designed as competitive inhibitors of prolyl-4-hydroxylase.[3] The latter enzyme is essential for the formation of a stable collagen trimer.[4] All general inhibitors of collagen, however, have potential for compromising the structural integrity of collagen-rich tissues such as skin, bone, and the vasculature. With this in mind, HOE 077 and Safironil were synthesized as inactive pro-drugs by amidation of their carboxyl groups. Their conversion to the active form requires oxidative deamidation, a function performed by the cytochrome P-450 family of heme proteins.[5] 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.[6] It also blocked fibrosis in female rats treated with carbon tetrachloride (CCl_4), with no measurable effects on normal tissues.[7] Although the data were consistent with the postulated mechanism of action of HOE 077, a conceptual problem remained, related to the drug's cellular target and site of action. Given the requirement for cytochrome P-450, the active form of HOE 077 will arise in hepatocytes. However, hepatocytes do not contribute significantly to fibrogenesis in liver injury. Rather, as shown by numerous laboratories, stellate cells (lipocytes, Ito cells, or fat-storing cells) are the principal source of liver collagen.[2,8–10] Therefore, if HOE 077 were to modulate stellate cell fibrogenesis by the mechanism proposed, its active metabolite(s) must undergo transfer from hepatocytes to stellate cells. Although electron microscopy has shown regions of close apposition of the two cell types,[11] there appear to be no specialized contacts. The mechanism of action of HOE 077 thus remains incompletely understood.

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A new understanding of the pathogenesis of hepatic fibrosis has emerged in recent years with characterization of the hepatic stellate cell and its activation as a central feature of the response to injury.[2,12,13] Activation denotes the conversion of resting stellate cells to myofibroblast-like cells and leads to a striking increase in extracellular matrix (ECM) production and expression *de novo* of smooth muscle α-actin. Thus, an agent that blocks activation will also reduce collagen expression at both the mRNA and protein levels. The possibility that HOE 077 acts in this manner is not formally excluded by any of the available data.

In this report, we confirm that HOE 077 down-regulates collagen production in animals given CCl_4. However, further analysis of parameters of stellate cell activation, including studies of primary cultures, indicate that its entire antifibrogenic effect is on activation rather than collagen protein synthesis, which is confirmed by the similar effect of a congener, Safironil, which has no inhibitory activity toward prolyl-4-hydroxylase. Because the initial studies of HOE 077 or Safironil involved female rats only, we used animals of both sexes, and the comparison proved interesting. There are subtle but significant differences in the up-regulation of collagen production in response to injury and clear differences in the sensitivity of the male and female animals, respectively, to both HOE 077 and Safironil. We conclude that the wound-healing response is sexually dimorphic, with stellate cell activation being more labile in female liver than in male liver and therefore more susceptible to modulation.

Materials and Methods

Induction of Fibrosis

Rats, either Sprague-Dawley (250 to 400 g) or Wistar (150 to 200 g) strains, were used. Fibrosis was induced with CCl_4 administered by gavage in an equal volume of olive oil every 5 days for either 3 weeks (short-course) or 6 weeks (long-course). For Wistar animals (males and females) and for the Sprague-Dawley males, the dose was 1 ml/kg body weight. However, in the Sprague-Dawley females, this produced excess mortality, and therefore a dose of 0.7 ml/kg was used. It induced fibrosis comparable to that in males by quantitative histological assessment using sirius red dye (Figure 1); by elution of the dye,[14] the amount of collagen in the male liver was increased 10% over control and in the female liver, 20%. Control animals received an equal volume of olive oil. Animals were sacrificed for study 5 days after the last dose. In one experiment, injury was induced by total ligation of the bile duct. This model has been well characterized with respect to the time course and extent of fibrogenesis.[9]

The antifibrotic compounds HOE 077 and Safironil were administered by gavage or provided in drinking water at a concentration of 1.5 mg/ml throughout the experiment unless indicated otherwise. In preliminary studies on the pharmacokinetics of HOE 077 and Safironil, a significant correlation was found between the concentration of drug in the animal's drinking water (0.5, 1.5, or 5.0 mg/ml) and peak serum levels (R = 0.999) and area under curve (AUC; 0 to 24 hours) (R = 0.993). Moreover, there were no differences between male and female rats with respect to serum concentration, the serum half-life of either compound, or AUC (0 to 6 hours; data not shown). Therefore, as a routine, water consumption was monitored to determine dosage. In some experiments as noted, the compounds were administered by gavage, with males receiving a higher dose than females, to ensure that the observed sex-specific effects of HOE 077 or Safironil *in vivo* were not due simply to inapparent differences in water consumption.

Preparation and Primary Culture of Hepatocytes and Stellate Cells

Hepatocytes were isolated by collagenase perfusion of the liver and purified by centrifugal elutriation, as described.[15] Preparations were 95 to 98% pure and >90% viable. For stellate cell isolation, the liver was dispersed by perfusion with collagenase and Pronase. The cell mixture was fractionated by centrifugation through a discontinuous gradient of metrizamide, and the upper layers were recovered and contained >95% stellate cells by cell number.[16] The cells were plated in 35-mm dishes (0.5×10^6 to 1.0×10^6 cells/dish) in a medium consisting of equal parts Dulbecco's modified Eagle's medium and Ham's F-12 with 20% v/v serum (horse/calf, 1/1). Co-cultures of hepatocytes and stellate cells were prepared by plating hepatocytes initially at one-half confluent density ($\sim 5 \times 10^5$ cells per 35-mm plate) in a modified Medium 199 with 5% calf serum.[15] Two hours later, after attachment and partial spreading of the hepatocytes, stellate cells were added as described above. Comparisons of HOE 077 and Safironil were performed with the same cell preparation, and preparations of male or female cells for an individual experiment were processed in parallel.

Analytical Procedures

RNA was extracted from freshly prepared or cultured stellate cells using TRI reagent (Molecular Research Center, Cincinnati, OH). Specific mRNAs were measured by RNAse protection assay, and cRNA probes were synthesized as described with [^{32}P]CTP and rat collagen I or human S-14 templates.[17] Results for collagen mRNA were normalized to S-14 mRNA, which encodes a ribosomal protein and varies minimally with liver injury.[17,18] The smooth muscle isoform of α-actin was measured by quantitative Western blot of extracts from cultured stellate cells, as described,[19] and equal amounts of protein extract were loaded. For both of these assays, intra-experimental variation was less than 10%. Studies with cultures were replicated with at least three different cell preparations. Typical results are shown. Synthesis of collagen was assayed as incorporation of [^{3}H]proline into protein digestible by highly purified collagenase.[20] PAC-1 cells (from rat pulmonary artery) were kindly provided by V. E. Koteliansky and grown in Dulbecco's modified Eagle's medium with 10% fetal calf serum.[21]

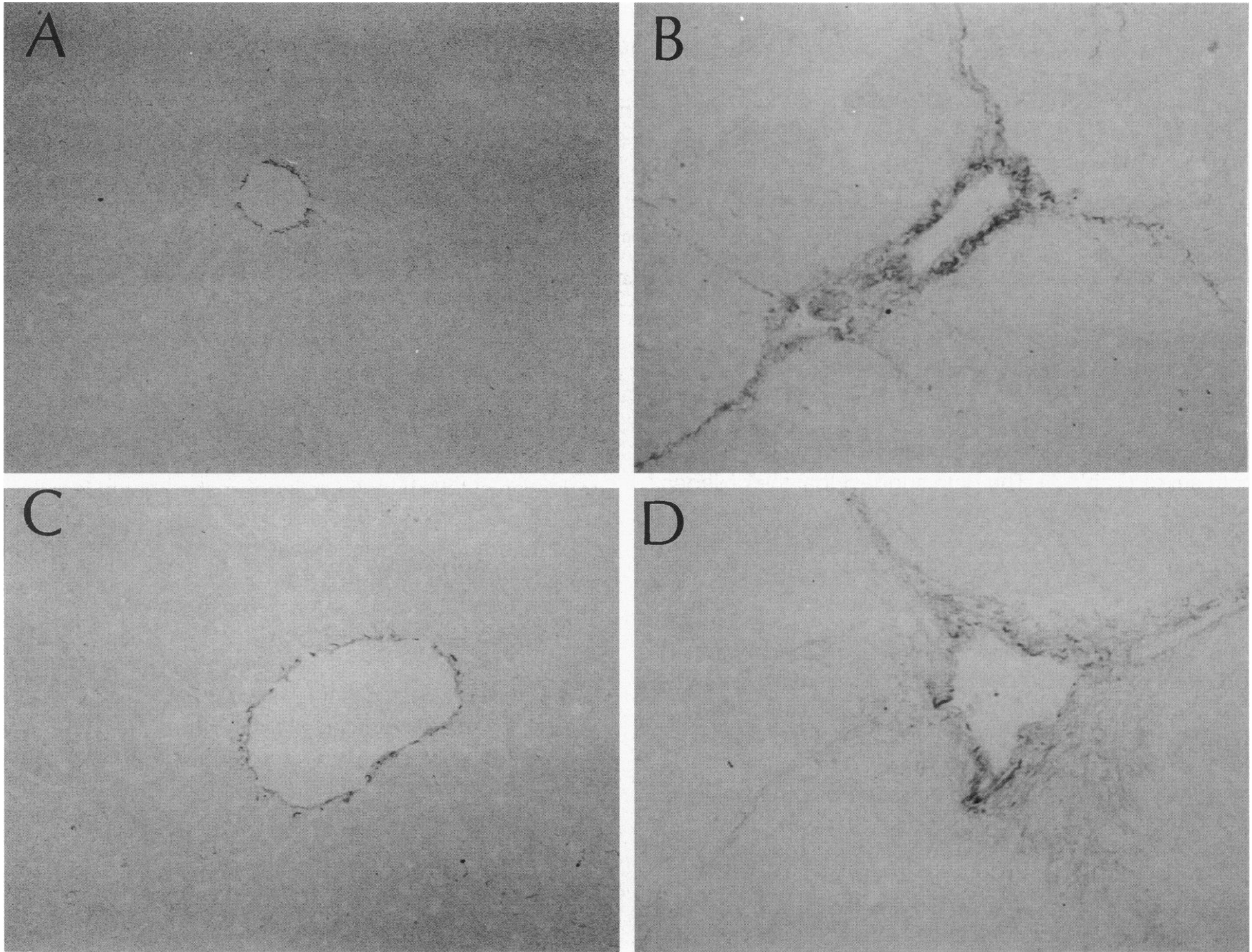


Figure 1. Histological assessment of fibrosis after a short course of CCl_4. Male or female Sprague Dawley rats received four doses of CCl_4 as described in Materials and Methods. At the time of sacrifice, a piece of liver was fixed in neutral buffered formalin, sectioned, and stained with sirius red[14]; representative areas are shown. Other samples from the same livers were analyzed for type I collagen mRNA (Figure 2).

Serum collagen fragments, procollagen III amino peptide and the 7S fragment of type IV collagen, were assayed as described.[22,23] Hepatic collagen was estimated from the hydroxyproline content of the liver, measured by the method of Palmerini et al.[24] The coefficient of variation for three independent experiments averaged 2.0% (range, 1.3 to 2.4%).

Statistical Methods

Differences were evaluated by nonparametric U-test. In the case of quantal responses, a χ^2 test was performed. Differences were considered significant at the level of $P < 0.05$.

Results

Injury Induction In Vivo: *Effect of the Antifibrotic Compounds HOE 077 and Safironil on Hepatic Collagen Synthesis*

Injury and early fibrosis were induced with short-term administration of CCl_4 to Sprague-Dawley rats as described in Materials and Methods. At the end of the experimental period, serum was taken for immunoassay of collagen IV 7S peptide and procollagen III amino peptide. In both males and females, CCl_4 induced a significant increase in circulating peptide. When HOE 077 or Safironil was given with the CCl_4, peptide levels decreased in female animals but not in males (Table 1). A second study examined two additional parameters of fibrosis, collagen I mRNA, and hepatic hydroxyproline, which indicates collagen protein content. To ensure that the observed effects were not peculiar to the Sprague-Dawley strain, Wistar animals were used. As shown in Table 2, both male and female animals responded to CCl_4 administration with a significant increase in hepatic collagen I mRNA. As in the preceding experiment, treatment with an antifibrotic agent (Safironil) reduced this significantly in females, relative to the corresponding treatment control, but not in males. The hepatic hydroxyproline level in treated female liver was not significantly decreased, but with a short course of CCl_4, animal-to-animal variation was wide. In a subsequent study with the long-course protocol, the expected decrease was observed (see below).

Table 1. Effects of HOE 077 and Safironil on Circulating Collagen Peptides during Short-Course CCl_4 Administration

Treatment group	Collagen IV/7S		PIIIP	
	Males	Females	Males	Females
Normal (n = 8)	15.10 ± 5.43	16.19 ± 2.02	2.61 ± 0.03	2.01 ± 0.32
CCl_4 (n = 8)	43.44 ± 13.28*	82.35 ± 17.03*	4.77 ± 0.36*	8.49 ± 2.39*
CCl_4 plus HOE 077 (n = 14)	36.45 ± 13.28	24.27 ± 0.73†	4.54 ± 0.79	4.67 ± 0.25†
CCl_4 plus Safironil	35.46 ± 8.31	27.36 ± 10.59†	4.22 ± 0.67	2.88 ± 0.87†

Collagen IV/7S, the 7S peptide of collagen IV (ng/ml serum); PIIIP, the amino-terminal peptide of collagen III (ng/ml serum).
*Significantly increased ($P < 0.05$) relative to the corresponding control.
†Significantly decreased ($P < 0.05$) relative to the corresponding values after CCl_4 alone.

The reduced collagen I mRNA in females pointed to an effect of the antifibrotic compounds at the pretranslational level. Also of interest was the fact that hepatic hydroxyproline was increased significantly only in males, despite an increase in circulating collagen peptides in both sexes (Table 1). The data suggest that the collagen synthetic response to CCl_4 is comparable in male and female liver but that newly synthesized collagen may be degraded more efficiently in the female liver or that the activated stellate cell phenotype reverts more readily in females.

In a next study, the period of treatment was extended to 6 weeks to test the observed differences between males and females beyond the period of acute fibrosis. Parameters of chronic injury were monitored, including serum bilirubin and ascites. As shown (Tables 3 and 4), in both males and females the elevation of serum alanine aminotransferase (ALT), bilirubin, and hepatic hydroxyproline as well as the incidence of ascites were similar. Under this protocol, treatment with HOE 077 or Safironil produced in females a significant reduction in hepatic hydroxyproline, which again was not observed in males. The number of females with ascites also decreased, although this reached statistical significance only for the group treated with HOE 077. None of these parameters improved in male animals despite an increased dose of the antifibrotic compounds. With the reduction of total hepatic collagen in the treated females, there was no change in the ratio of hydroxyproline to proline, arguing against an effect of the drug on proline hydroxylation (data not shown).

Given that activated stellate cells are the principal source of collagen in liver injury, it seemed likely that the differing response of male and female liver was referable to this cell type. This hypothesis was confirmed by inducing injury and then isolating stellate cells and quantitating the level of collagen I mRNA in the fresh preparations. After administration of CCl_4, collagen I mRNA in both males and females was markedly increased over controls (Figure 2). With concomitant administration of HOE 077 or Safironil, it was reduced. After normalization to S-14 mRNA (to correct for RNA loading), the effect of both antifibrogenic drugs was substantially greater in female

Table 2. Effect of Safironil on Collagen Expression during Short-Course CCl_4 Administration

Treatment group	Collagen I mRNA		Hydroxyproline	
	Males	Females	Males	Females
Control (n = 6)	100 ± 21	100 ± 12	177 ± 26	195 ± 13
CCl_4 (n = 12)	544 ± 203*	196 ± 187*	276 ± 39*	199 ± 17
Safironil (n = 6)	100 ± 16	100 ± 63	193 ± 8	211 ± 38
CCl_4 plus Safironil (n = 12)	391 ± 60	43 ± 64†	267 ± 60*	289 ± 258

Safironil was administered by gavage three times daily. The dose for males was 30 mg/kg and for females was 3 mg/kg. Results are expressed as mean ± SD. For collagen I mRNA, the level was quantitated by RNAse protection assay (see Materials and Methods). The data indicate the percent change from the corresponding control, set at 100%. For hydroxyproline, total hepatic collagen was measured as hydroxyproline in protein (ng/mg liver tissue, wet weight).
*Increase is significant relative to corresponding control ($P < 0.05$).
†Decrease is significant ($P < 0.05$) relative to CCl_4.

Table 3. Effect of HOE 077 on Hepatic Injury and Collagen Content after Long-Course CCl_4

Treatment group	Bilirubin (μmol/L)		ALT (U/L)		Hydroxyproline (ng/mg)		Ascites (%)	
	Males	Females	Males	Females	Males	Females	Males	Females
Control (10 M, 10 F)	3.2 ± 0.6	2.05 ± 0.4	46.6 ± 8.4	41.7 ± 6.2	226 ± 38	178 ± 26	0	0
CCl_4 (75 M, 100 F)	10.4 ± 8.6*	8.5 ± 8.1*	132 ± 73*	141 ± 125*	768 ± 423*	666 ± 491*	14.1*	21.6*
CCl_4 plus HOE 077 (90 M, 75 F)	13.1 ± 7.1	7.0 ± 7.2	130 ± 39	131 ± 134	837 ± 454	479 ± 303†	12.7	8.1†

HOE 077 was given in drinking water; male animals received 120 mg/kg, female animals 90 mg/kg. The data represent mean ± SD. The number of male (M) and female (F) animals studied is shown in parentheses. Bilirubin is total in serum; ALT is alanine aminotransferase in serum; hydroxyproline is total hepatic collagen measured as hydroxyproline (ng/mg liver tissue); and ascites is the percentage of animals with visible ascites.
*Value is significantly elevated ($P < 0.05$) compared with corresponding control.
†Value is significantly reduced ($P < 0.05$) relative to CCl_4.

Table 4. Effect of Safironil on Hepatic Injury and Collagen Content After Long-Course CCl_4

Treatment group	Bilirubin (μmol/L)		ALT (U/L)		Hydroxyproline (ng/mg)		Ascites (%)	
	Males	Females	Males	Females	Males	Females	Males	Females
Control (10 M, 15 F)	2.5 ± 0.4	2.1 ± 0.8	41 ± 6	42 ± 10	214 ± 20	173 ± 31	0	0
CCl_4 (70 M, 96 F)	13.1 ± 11.2*	5.3 ± 5.1*	139 ± 20*	105 ± 60*	1054 ± 603*	640 ± 458*	24.2*	16.9*
CCl_4 plus Safironil (90 M, 96 F)	12.2 ± 9.4	4.5 ± 4.3	125 ± 47	97 ± 50	1028 ± 564	516 ± 314†	26.6	8.8

Safironil was given by gavage three times daily; male animals received a daily dose of 9 mg/kg, female animals 3 mg/kg. The data represent mean ± SD. The number of male (M) and female (F) animals studied is shown in parentheses. Bilirubin is total in serum; ALT is alanine aminotransferase in serum; hydroxyproline is total hepatic collagen measured as hydroxyproline (ng/mg liver tissue); and ascites is the percentage of animals with visible ascites.

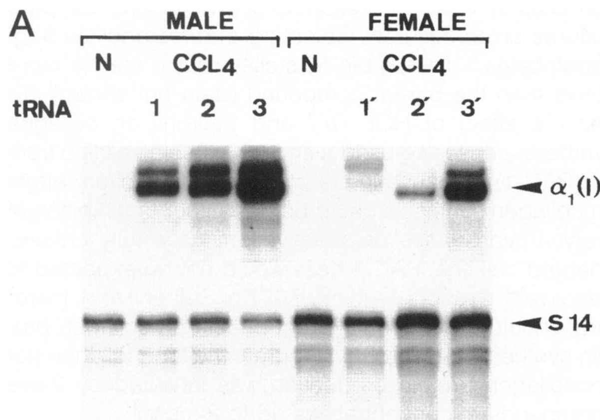
*Value is significantly elevated ($P < 0.05$) compared with corresponding control.

†Value is significantly reduced ($P < 0.05$) relative to CCl_4.

cells than in male cells, paralleling the studies of whole-liver mRNA (Table 2).

Culture Activation of Stellate Cells: Effects of HOE 077 and Safironil

Stellate cells, isolated from normal animals and plated on culture plastic, during the initial 3 to 5 days *ex vivo* spontaneously acquire 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.[12] Its salient features include *de novo* production of smooth muscle α-actin as well as markedly increased expression of collagen I. Before testing the effect of the antifibrotic compounds on stellate cell activation in culture, toxicity studies were performed with HOE 077 and Safironil. No effect was seen 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. These findings are similar to those reported previously for hepatocytes in primary culture.[6] The antifibrogenic effect of HOE 077 and Safironil then was examined with early primary cultures of stellate cells. Both drugs reduced expression of mRNA for type I collagen in a dose-dependent manner, and the effect was observed in male as well as female cells (Figure 3). However, the response of female cells was greater in magnitude, particularly at the lowest concentration of drug. By immunohistochemistry, type I collagen protein decreased in parallel (data not shown). To exclude the possibility that HOE 077 and Safironil were acting selectively on collagen I expression, an indepen-



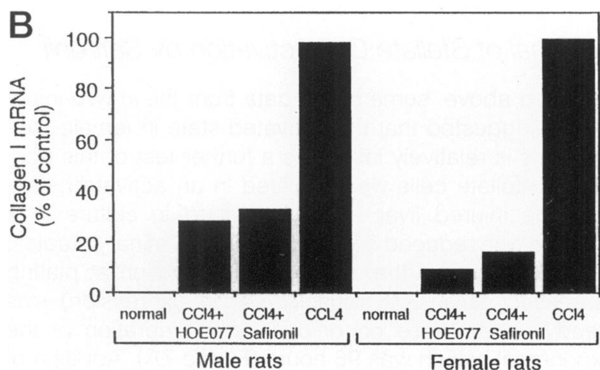


Figure 2. Effect of HOE 077 or Safironil on expression *in vivo* of collagen I mRNA by hepatic stellate cells. **A:** Animals were treated with vehicle only (N, normal), with CCl_4 plus HOE 077 (**lanes 1** and **1'**), CCl_4 plus Safironil (**lanes 2** and **2'**), or CCl_4 only (**lanes 3** and **3'**). The HOE compounds were added to drinking water (1.5 mg/ml). As an RNA control, yeast tRNA was used in place of stellate cell RNA. Treatment with either HOE compound alone was no different from normal (data not shown). **B:** Quantitation of the RNAse protection assay, showing the effect of HOE 077 or Safironil relative to treatment controls (CCl_4 alone). S-14 mRNA was scored in the same samples as an invariant internal mRNA. Autoradiographic bands were quantitated by scanning densitometry and corrected for mRNA loading by S-14 measurement. A representative result is shown.

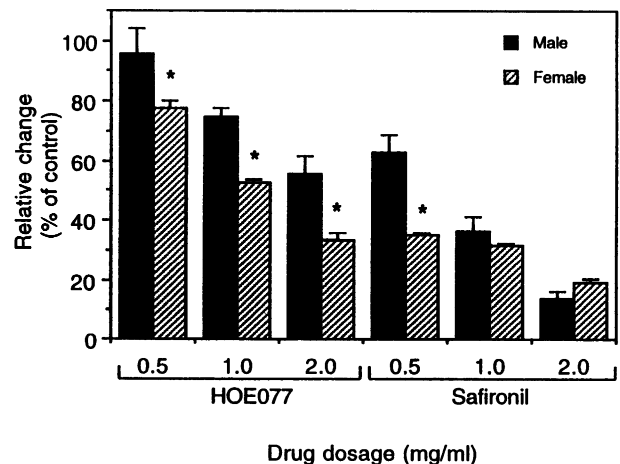


Figure 3. Collagen I mRNA in stellate cells in primary culture. Stellate cells were isolated from normal male or female rat liver and placed in primary culture, where they underwent activation over a period of 5 days. The indicated concentrations of HOE 077 or Safironil were added on day 2, and their effect was evaluated by RNAse protection assay, as in Figure 2. The data are expressed relative to control cells, set at 100% (n = 4). *P >0.05, male *versus* female at a given concentration of HOE 077 or Safironil.

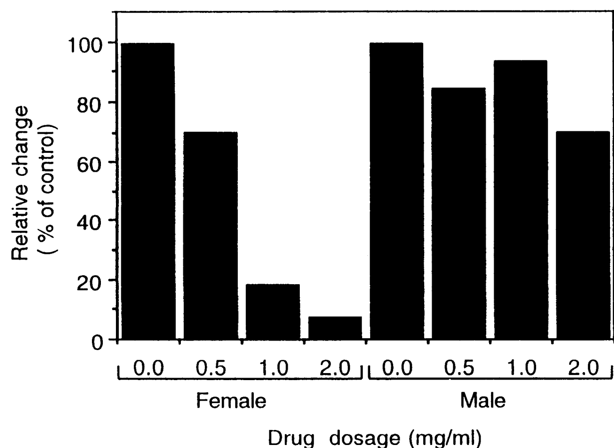


Figure 4. Expression of smooth muscle α-actin by stellate cells cultured with HOE 077. Stellate cells were isolated from normal male or female liver and placed in culture, as in Figure 3, with or without HOE 077 at the concentrations indicated in mg/ml. After 5 days, protein extracts were prepared and analyzed for smooth muscle α-actin by quantitative immunoblot. The bands were scanned and quantitated. Results of a representative experiment are shown.

dent parameter of stellate cell activation, smooth muscle α-actin, was also monitored. Both HOE 077 (Figure 4) and Safironil (Figure 5) reduced the expression of this protein, and again cells from females were more sensitive to either compound than were cells from males. The data provide direct evidence that the antifibrogenic effect of HOE 077 or Safironil involves down-regulation of stellate cell activation and that the differential sensitivity of the female to these drugs also involves the stellate cell.

Mechanism of Action of HOE 077

Although both antifibrotic compounds clearly modulate collagen synthesis by down-regulating stellate cell activation, HOE 077 (but not Safironil) conceivably could act also by inhibiting prolyl-4-hydroxylase, at least *in vivo*. If the drug indeed is metabolized in hepatocytes to compounds that include competitive inhibitors of prolyl-4-hydroxylase, such inhibitors could undergo transfer to stellate cells. We evaluated this possibility by comparing pure cultures of stellate cells with hepatocyte stellate cell co-cultures. HOE 077 exerted similar effects on pure cultures or co-cultures, indicating that metabolites from hepatocytes,[6] if available to stellate cells, are no more active than the parent compound (data not shown). To test the effect of HOE 077 and Safironil on collagen synthesis *per se*, we used a smooth muscle cell line from rat (PAC-1), because in stellate cells an inhibitory effect on collagen synthesis could be due either to inhibition of prolyl-4-hydroxylase or deactivation. As a fully culture-adapted cell line, PAC-1 cells would not be expected to deactivate in response to HOE 077 or Safironil and, therefore, should register only direct effects on collagen protein synthesis. As shown in Figure 6, neither collagen nor noncollagen protein production was inhibited by these compounds at concentrations up to 2 mg/ml.

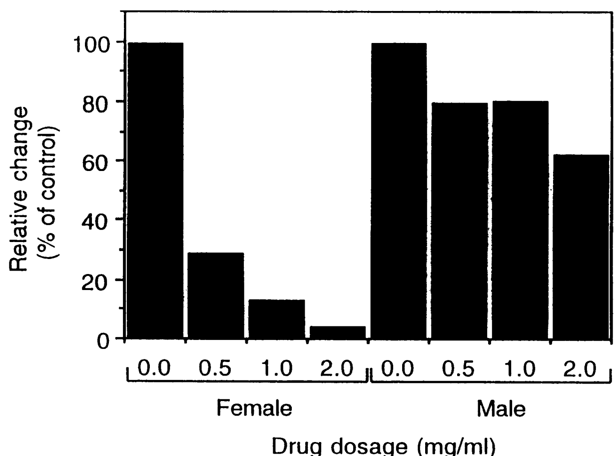


Figure 5. Expression of smooth muscle α-actin by stellate cells cultured with Safironil. The experiment was identical to that in Figure 4, with the concentrations of Safironil indicated in mg/ml.

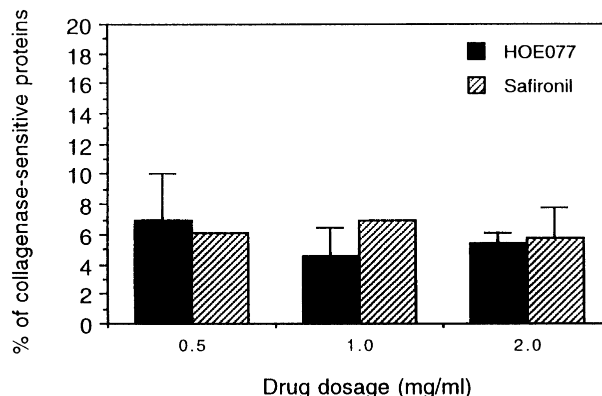


Figure 6. Effect of HOE 077 or Safironil on collagen protein synthesis by smooth muscle cells in culture. PAC-1 cells were plated and, when subconfluent, changed to medium containing the indicated concentrations of drug and radioproline (see Materials and Methods). Collagen and noncollagen protein synthesis were quantitated as described.[22]

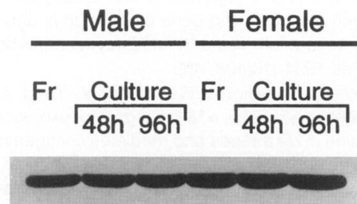
Reversal of Stellate Cell Activation by Safironil

As noted above, some of the data from the *in vivo* injury model suggested that the activated state in female stellate cells is relatively labile. As a further test of this possibility, stellate cells were isolated in an activated state from the injured liver and established in culture in a medium with reduced serum (5%). In preliminary studies, it was determined that the level of activation at plating (assessed as smooth muscle α-actin expression) was steady under these conditions for the duration of the experiment, which was 96 hours (Figure 7A). Addition of Safironil to the culture medium produced a striking decrease in smooth muscle α-actin in the female cells, and an effect in cells from males was observable but relatively minor (Figure 7B).

Discussion

In these studies, the mechanism of action of both HOE 077 and Safironil is shown to involve largely, if not exclu-

A.



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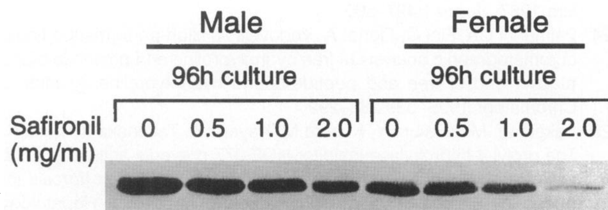

Figure 7. Reversal of stellate cell activation by Safironil. Hepatic injury was induced in male or female rats by ligation of the biliary duct. Five days later, activated stellate cells were isolated and plated in medium containing 5% serum. **A:** The stability of *in vivo* activation under these culture conditions was assessed by extracting fresh cells (Fr) or cells from the same preparation maintained in culture for 48 or 96 hours. The level of smooth muscle α-actin was measured by immunoblot (see Materials and Methods). **B:** In parallel experiments, the medium was changed at 24 hours to one with the indicated concentration of Safironil. After an additional 72 hours, the cultures were extracted for quantitation of smooth muscle α-actin. A representative result is shown.

sively, stellate cell activation. The specificity of HOE 077 for the liver in the CCl_4 model was believed initially 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 HOE 077 is targeted to the myofibroblast response in wound repair. Thus, the reported liver specificity of these drugs[7] may reflect largely the fact that, in CCl_4-mediated injury, the repair process is centered on the liver. This raises the interesting possibility that compounds such as HOE 077 and Safironil may target wound repair beyond the liver and have therapeutic potential for a variety of fibrosing diseases. Their molecular mechanism of action remains to be determined. With respect to the CCl_4 model of liver injury, interference with the toxic effect of the agent would be a trivial mechanism. This was considered previously and excluded,[7] and testing with a completely different model of liver injury produces similar results.[25] A more detailed understanding of the way in which HOE 077 acts awaits further study of stellate cell activation, a program that involves products of inflammation and various cytokines, including transforming growth factor-β, tumor necrosis factor-α, and platelet-derived growth factor.[2] Changes in the extracellular matrix also play a role; endothelial cell production of a specific splice variant of fibronectin (the EIIIA-containing form) has been shown to stimulate stellate cell activation.[17] Finally, changes in calcium flux also occur[26] and may be of particular interest in that HOE 077 and its major metabolites, as pyridine dicarboxylates, resemble calcium channel blockers.

We show that these inhibitors of stellate cell activation have particular efficacy in females and thus extend the data of Sakaida and colleagues, who studied male animals only.[25] The sex-specific difference is seen not only *in vivo* but also in cultures of male or female cells never exposed to CCl_4, and some of the data suggest that deactivation is affected preferentially. Stellate cell activation and its associated fibrogenesis are dynamic and reversible. If the injury is eliminated, activation subsides and the excess extracellular matrix deposited in response to the injury is degraded and resorbed.[27,28] If the injury persists, the neomatrix undergoes organization, developing parallel arrays of cross-linked collagen fibrils, and becomes relatively resistant to degradation.[29] Both the 3-week and 6-week CCl_4 protocols induce injury that is completely reversible after the stimulus is withdrawn.[28] Where the stimulus is persistent, as in chronic viral hepatitis, the balance between activation and deactivation is determined by both soluble factors, such as cytokines, and the extracellular matrix in the pericellular environment.[2] It is currently unknown whether the expression of these postulated regulatory factors differs between males and females or whether any is modulated by ovarian steroids. According to a preliminary communication, estradiol suppresses fibrosis in rats given dimethylnitrosamine.[30] Also reported is a beneficial effect of estradiol in postmenopausal women with chronic hepatitis, which is intriguing, if anecdotal, evidence that human liver fibrogenesis also may be modulated by estrogen.[31] Apart from the liver, a considerable literature exists regarding atherogenic vascular injury, which could be viewed as wound repair of the arterial intima[32–34] and is estrogen responsive.[35–37]

The present findings may be informative with respect to the over-representation of males among patients with chronic fibrosing liver disease and its complications. Clinical experience and death statistics support the view that cirrhosis is largely a disease of males (with the exception of the classically autoimmune diseases, primary biliary cirrhosis and chronic autoimmune hepatitis). Chronic viral hepatitis (B or C) appears on a clinical basis to cause more inflammation[38] and progress more rapidly in men than in women.[39,40] Sex-related differences are most striking with respect to the incidence of hepatocellular carcinoma, a cancer closely associated with fibrosis or cirrhosis. In patients infected with hepatitis B, age-specific mortality from hepatocellular carcinoma is three-fold greater in men than in women over the age of 45.[41] Similar figures are emerging for the incidence of this cancer in hepatitis C.[42] Alcohol consumption does not appear to be a confounder,[43] particularly as the disease-causing level of alcohol intake is lower for women than for men.[44] Finally, these data may explain conflicting results on the efficacy of colchicine for preventing fibrosis.[45] In the one favorable study, the treatment group contained a larger proportion of females than did the control.[46] A subsequent study of predominantly male patients with chronic hepatitis B showed no effect of this drug.[47]

In summary, we have demonstrated that the novel compounds HOE 077 and Safironil reduce fibrogenesis in liver injury by inhibiting stellate cell activation. As such, they target inflammation and wound repair and mitigate concern for the potential side effects of direct inhibitors of collagen protein synthesis. The studies have elucidated also an interesting sexual dimorphism in myofibroblast metabolism that may be relevant generally to wound healing, with implications for the therapy of chronic fibrosis in males and females, respectively.

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