Hepatobiliary Damage and Changes in Hepatic Gene Expression Caused by the Antitumor Drug Ecteinascidin-743 (ET-743) in the Female Rat

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ABSTRACT

Ecteinascidin-743 (ET-743) is a novel marine-derived anticancer drug with clinical activity in soft tissue sarcoma and ovarian cancer. Reversible transaminisits and subcircular cholangitis have frequently been described in patients who receive ET-743. To facilitate understanding of this adverse effect and help design suitable therapeutic rescue strategies, we characterized the hepatic effects of ET-743 in rats. Female rats received ET-743 (single dose, 40 μg/kg) i.v., and liver changes were assessed from 6 h up to 3 months after dosing by histopathology, immunohistochemistry, electron microscopy, hepatic and plasma biochemistry, and DNA microarray analysis. At 24 h posttreatment and beyond, livers displayed degeneration and patchy focal necrosis of bile duct epithelial cells associated with mild inflammation followed by fibrosis. Sporadic and focal zones of hepatic necrosis and hemorrhage were observed from day 2 onward, although the majority of hepatocytes appeared normal as judged by electron microscopy. Pathological alterations persisted up to 3 months after dosing. Plasma levels of total bilirubin were elevated up to 7-fold over those in untreated rats from day 2 onward and returned to control values by day 24. Activities of alkaline phosphatase and aspartate aminotransferase in plasma were elevated for 2 and 3 months, respectively. Activities of the hepatic microsomal drug-metabolizing enzymes cytochrome P-450 A1/2, CYP2E1, and CYP3A2 were decreased. DNA microarray analysis of livers from ET-743-treated animals showed a dramatic increase in the expression of ATP binding cassette transport genes Abcb1a and Abcb1b, which impart resistance to anticancer drugs, and of Cdc2a and Ccnd1, the rodent homologues of human cell cycle genes CDC2 and cyclin D1, respectively. The cell cycle gene expression changes mirrored ET-743-induced increases in liver weight and Ki-67 labeling of liver nuclei. The results suggest that the toxicity exerted by ET-743 in the rat liver is a consequence of biliary rather than hepatocellular damage and that it is accompanied by a wave of mitogenic activity, which may be driven by the transcriptional increase in Cdc2a expression.

INTRODUCTION

Ecteinascidin 743 (ET-743) is a tetrahydroisoquinoline alkaloid isolated from the marine tunicate Ecteinascidia turbinata that possesses potent antineoplastic activity against a variety of human tumor xenografts grown in athymic mice in vivo, including melanoma and ovarian and breast carcinoma (1–3). In clinical Phase I studies of ET-743, promising responses were observed in patients with sarcoma and breast and ovarian carcinoma (4–7). The drug is currently under investigation in Phase II trials in cancer patients with a variety of neoplastic diseases. Patients who received ET-743 by prolonged infusions over 24–72 h experienced myelosuppression and frequently acute (but reversible) transaminisits and subcircular cholangitis characterized by increases in ALP and/or bilirubin. Preclinical acute toxicity studies conducted in mice, rats, dogs, and monkeys demonstrated liver toxicity as an important side effect of ET-743, as evidenced by an increase in plasma levels of liver-specific enzymes and pathological manifestations of cholangitis (8). In these studies, the female rat was identified as the species with the highest susceptibility to the hepatotoxic potential of ET-743 and showed treatment-related blood chemistry alterations not unlike those accompanying hepatotoxicity seen in the clinical trials of ET-743 (4–7).

The mechanism of antineoplastic action of ET-743 is not yet fully understood, and the mechanisms responsible for its hepatotoxic potential are unknown. The drug has been shown to bind to the minor groove of DNA and alkylate the N2 position of guanine at specific sequences (9). At pharmacologically active concentrations, ET-743 caused perturbations of the cell cycle with a decrease in the rate of progression of S-phase cells toward G2 and a prolonged blockade in G1-M (10). The drug inhibited the binding of several transcription factors to DNA (11–13), prominent among them nuclear factor Y (12, 13), which activates transcription of some cell cycle genes. Therefore, the perturbations of the cell cycle machinery exerted by ET-43 may be caused, at least in part, by its ability to inhibit nuclear factor Y transactivation. Cells deficient in nucleotide excision repair ability were resistant to the cytotoxic potential of ET-743 (14), a finding that suggests that the mode of cytotoxicity of ET-743 is distinctly different from that of other DNA-interactive cytotoxicants.

Taken together, these results characterize ET-743 as a new drug with anticancer activity and a novel, albeit still somewhat obscure, mechanism of antineoplastic activity. In light of the paucity of information available on details of its hepatotoxic potential, we investigated its hepatotoxicity in vivo in the female rat, the rodent species that seems to be most sensitive to this adverse effect of ET-743, by detailed analysis of ET-743-induced changes in liver pathology, biochemistry, and accompanying gene expression profiles. The overall aim of the study was to increase our understanding of the hepatotoxicity of ET-743 and identify biological events underlying the observed hepatic alterations that may ultimately be exploited by novel therapeutic regimens or drug combinations designed to alleviate this adverse effect.

MATERIALS AND METHODS

Animals and Treatments. ET-743 was obtained from PharmaMar SA (Madrid, Spain), the drug manufacturer. Experiments were conducted as stipulated by Project License 80/1250 granted to the MRC Toxicology Unit by the United Kingdom Home Office. Groups of four female Wistar rats (230–260 g) received either a single dose of ET-743 (40 μg/kg) i.v. or the vehicle (water) via the lateral tail vein. In a preliminary dose-finding orientation experiment, ET-743 administered at 75 μg/kg, the maximum tolerated dose in the female rat, elicited severe toxicity, resulting in mortality. Therefore the dose was reduced to 40 μg/kg, which caused toxicity but avoided mortality. This dose is close to 1500 μg/m2 (approximately 38 μg/kg), the dose recommended to be
infused in Phase II studies in patients (5). Animals were killed 6, 12, or 24 h; 2, 3, 6, 12, 24, or 48 days; or 3 months after administration. Blood was obtained by cardiac puncture, and blood cells were separated from plasma by centrifugation. Slices of liver were excised and fixed in buffered formalin (10%). The remaining liver tissue was homogenized in ice-cold 50 mM Tris-KCl buffer containing 0.25 M sucrose (pH 7.4). For isolation of liver microsomes, the homogenate was centrifuged (10,000 x g, 20 min, 4°C), and the supernatant was then removed and spun at 100,000 x g for 60 min at 4°C (15). The microsomal pellet was resuspended in fresh buffer and recentrifuged (100,000 x g) for an additional 60 min. The resulting pellet was suspended in 0.25 M phosphate buffer containing 30% glycerol, stored at –80°C, and thawed before analysis. The Bradford assay was used to determine protein concentration.

Selected tissues other than liver (including stomach, small intestine, thymus, spleen, heart, lungs, kidneys, and bone marrow) from rats killed at periods of up to 3 days after treatment were taken for histological examination.

**Histopathology and Immunocytochemistry.** Tissues were fixed in neutral buffered formalin and embedded in paraffin wax. Sternum samples were decalcified for examination of bone marrow cellularity. Sections (5-µm thick) were cut and stained with H&E. Selected hepatic sections were stained with van Gieson’s stain for collagen.

Ki-67 was demonstrated in sections of formalin-fixed, paraffin wax-embedded liver tissue at all time points between 6 h and 12 days after dosing. Sections were dewaxed in xylene, immersed in water, and microwaved in citrate buffer (pH 6.0) for 30 min at 700 W. A primary polyclonal rabbit antibody to a 1086-bp Ki-67 motif-containing cDNA fragment (NCL-Ki67p; Novacrafta) was applied at a dilution of 1:500 for 3 h at room temperature. Normal rabbit immunoglobulin (X0903; DAKO) was used as a negative control. The primary antibody was detected with the DAKO Duet System (K0492; DAKO). Positive nuclei were visualized using 3,3′-diaminobenzidine tetrahydrochloride, and sections were lightly counterstained with hematoxylin.

The total number of nuclei/unit area was calculated by counting the number of nuclei in a rectangular frame (0.32 x 0.225 mm) using the x40 Diaplan microscope objective on the H&E-stained sections, and counting was repeated n times in a rectangular frame (0.32 x 0.225 mm) for the determination of the total number of nuclei/unit area. The total number of nuclei/unit area was calculated by counting the number of nuclei in a rectangular frame (0.32 x 0.225 mm) using the x40 Diaplan microscope objective on the H&E-stained sections, and counting was repeated in 10 randomly chosen frames. The proliferation index was calculated as the number of Ki-67-stained nuclei/1000 hepatocyte nuclei.

The presence of α-smooth muscle actin was demonstrated using a mouse monoclonal antibody (clone 1A4, IgG2a, DAKO M 0851) against the NH2-terminal decapeptide of human α-smooth muscle actin. Sections were pre-
treated as described above, and the primary antibody was applied at a dilution of 1:100 for 3 h at room temperature). Mouse IgG2a-negative control antibody (X0943; DAKO) was used as control. The primary antibody was detected as described above. The peroxidase label was visualized using the VIP substrate kit (SK-4600; Vector Laboratories) followed by a light hematoxylin counterstain.

**Electron Microscopy.** Livers were fixed by vascular perfusion with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) and stored overnight (4°C) in the fixative. Slices (<1-mm thick) were postfixed with 1% osmium tetroxide/1% potassium ferrocyanide, stained en bloc with 5% uranyl acetate, and embedded in Taab epoxy resin (Taab Ltd.). Ultrathin sections were examined unstained or after staining with lead citrate and/or uranyl acetate.

**Measurement of Liver Enzymes, Bilirubin, and Cytochrome P450 Isozymes, and Cell Cycle Distribution.** Plasma levels of ALP, AST, and total plasma bilirubin were measured using commercially available kits and established protocols (Sigma, St. Louis, MO). Total microsomal cytochrome P450 protein content was determined by the method of Adams et al. (15), and activities of liver microsomal enzymes associated with cytochrome P450 isoforms CYP1A1/2, CYP2E1, and CYP3A2 were measured according to Burke et al. (16), Carlson (17), and Chang and Yeung (18) using ethoxysreorbin, 4-nitrophenol, and 7-benzyloxy-4-trifluoromethylcoumarin, respectively, as substrates.

Cell cycle analysis on liver nuclei from control and ET-743-treated rats was performed as described previously using fluorescence-activated cell-sorting scan (19).

**Microarray Studies.** In a separate study, female Wistar rats were treated as described above with ET-743, and the liver of each treated rat was paired with liver from an age-matched vehicle-treated control rat. Groups of three ET-743-treated rats paired with three untreated rats were killed 6 h and 1, 2, 3, 6, and 24 days after dosing. Analysis of hepatic gene expression was carried out using cDNA microarrays containing approximately 4700 hybridizable mouse expressed sequence tags derived from IMAGE clones obtained from Research Genetics (Huntsville, AL) or from the MRC Human Gene Mapping Project. At each time point, one array was used for each pair of rats, and the individual RNAs were labeled with Cy3 or Cy5. The labels were reversed for subsequent hybridizations. Microarray preparation, RNA labeling, and hybridization were performed as described previously by Turton et al. (20). The clones of interest were sequenced to confirm identity. Where differential expression was determined, sequence homology with the appropriate rat gene was assessed to confirm that cross-hybridization between the species could occur. Pixel intensity for both the features and the background was assessed using GenePix software (Axon Instruments, Union City, CA) version 3.0.6 and an Axon 4000A scanner. The data were normalized and processed to a final measure of differential gene expression, quantitated as a ratio of ET-743-treated/control, as described previously (20), using ConvertData version 3.40.c. Clustering analysis was performed by determination of the principal components of the score data (20) using SIMCA-P (Umetrics, Bracknell, United Kingdom). The expression of genes that were significantly altered with reference to all of the other genes on the same array were used for a principal components analysis. Each microarray was kept as a separate entity for this analysis, as were the replicated clones on the microarrays. For the hepatic genes, the expression of which was consistently up- or down-regulated by ET-743 (Cdc2a, Cend1, Abcd1a, Abcb1b, and Car 3, see “Results”), homology between the mouse and the rat was established to be 98% or greater. Data supplementing the microarray results shown in Figs. 4 and 5 are contained on the World Wide Web.

**Statistical Analysis.** Significance of differences was assessed using one-way ANOVA followed by Tukey’s post hoc test.

**RESULTS**

**Effect of ET-743 on Liver Pathology.** To study liver changes induced by ET-743, female rats received a single dose of ET-743 (40 µg/kg) i.v. Within 4–6 days after dosing, animals lost 15% of their body weight, compared with controls. Whereas livers obtained 6 and 12 h after ET-743 administration did not show signs of pathological change, the epithelial cells lining the larger bile ducts in all treated animals displayed focal degenerative alterations at 24 h after treatment. By days 2 and 3, these changes involved many bile ducts in all treated animals. Livers were characterized by degeneration and patchy focal necrosis of bile duct epithelial cells associated with a modest acute inflammatory infiltrate on day 2 and thereafter (Fig. 1, A and B). A day later, these alterations were more pronounced and accompanied by increased inflammation (Fig. 1C) and early signs of regeneration of the epithelial cells characterized by enlarged cell cytoplasm and large irregular nuclei with occasional mitoses. Six and 12 days after treatment with ET-743, bile ducts were surrounded by dense, poorly cellular, concentric fibrosis (sclerosis) (Fig. 1E) and by mesenchymal cells that stained for α smooth muscle actin (Fig. 1F). After 24, 48, and 92 days, the inflammation in the portal tract had diminished substantially, although periductal fibrosis remained prominent (Fig. 1G). Blood vessels appeared histologically within normal limits. In some treated rats, rounded focal zones of hepatic necrosis and hemorrhage were observed from day 2 onward, with little or no inflammation (Fig. 1D). These alterations persisted up to 3 months. Some focal pigmentation was seen around zones of necrosis at time points >24 h after administration of ET-743. Apart from these foci of necrosis, most hepatocytes showed relatively little alteration on light microscopic examination, except that mitotic activity was evident 3 days after treatment.

Electron microscopy showed that within 2 days after administration of ET-743, there was focal injury including cell-sorting changes in the epithelium of bile ducts, often resulting in the liberation of

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4 http://www.igmp.mrc.ac.uk

5 http://www.le.ac.uk/cmht/microarray_lab/Home.
cell debris into the lumen (Fig. 2A). Most of the liver remained largely normal. The foci of hepatic necrosis (oncasis) and hemorrhage did not correlate with particular regions of the lobule. The tight junctions isolating bile canaliculi, even between severely damaged cells, were usually intact. Despite the presence of numerous erythrocytes within these foci, there was little or no inflammation. Abnormal mitochondria were evident in many necrotic cells and probably represented a phenomenon secondary to the necrosis. Hepatocytes immediately surrounding the focal lesions tended to have rather more smooth endoplasmic reticulum than controls, and it was dispersed throughout the cytoplasm rather than aggregated in large clumps (Fig. 2B). Apparently normal hepatocytes were immediately adjacent to affected cells. Venous endothelial cells were often distended in affected zones, which did not seem to be a primary injury because intact endothelial cells were present overlying oncotic hepatocytes. The endothelial lining of sinusoids was largely undamaged, but some sinusoids were blocked by erythrocytes, neutrophils, platelets, and luminal fibrin.

As far as ET-743-induced extrahepatic damage is concerned, there was a degenerative change in the small intestine to cells deep within the intestinal crypts as early as 6 h after treatment. This degeneration was visible up to 24 h but had disappeared by day 3. Pancreatic ducts in the head of pancreas were not systematically sectioned, but in sections in which they were visible, their appearance was entirely normal. There was also mild loss in cellularity of bone marrow at 12, 24, and 72 h. The thymus tissue examined showed little or no definite evidence of loss in cellularity, and the appearance of the other organs investigated was within normal limits.

In an orientation experiment, male rats were investigated instead of females. In male rats, 40 μg/kg ET-743, the dose that had elicited the changes described above in female animals, caused only slightly irregular bile duct epithelia and sparse degenerate biliary cells 3 days after dosing and additional mild peribiliary fibrosis at day 12. Nevertheless, 80 μg/kg ET-743 was profoundly toxic in male rats and caused changes to bile ducts and necrosis in liver cells that mimicked...
Bilirubin started to increase in the lumen. The hepatocyte in B, which was found near a zone of necrosis, displays slightly more smooth endoplasmic reticulum than hepatocytes in control animals but is otherwise essentially within normal limits. Bars, 10 μm. The micrographs are representative of four separate animals. For details of electron microscopy, see “Materials and Methods.”

in character and extent the liver pathology observed in female rats after administration of 40 μg/kg ET-743.

**Effect of ET-743 on Liver Biochemistry.** Plasma levels of total bilirubin started to increase >24 h after female rats had received ET-743, and at 3 days, levels were elevated 7-fold over controls (Fig. 3A). This elevation persisted until at least 12 days and returned to basal levels by day 24. Levels of liver enzymes ALP and AST were significantly raised from 48 h after administration of ET-743 onward (Fig. 3A). Maximal elevation of ALP and AST levels was approximately 2-fold over control levels by day 3, and the rise in AST activity persisted up to 3 months after administration, the furthest time point examined. In male rats, plasma levels of bilirubin, ALP, and AST were not affected by the dose of ET-743 (40 g/kg) that elicited marked elevation in females. However 80 μg/kg ET-743 increased the biochemical markers of hepatotoxicity in male rats as dramatically as seen with the lower dose in females.

ET-743 also affected the activities of hepatic microsomal drug-metabolizing cytochrome P450 enzymes (Fig. 3). Activities of CYP3A2, CYP1A1/2, and CYP2E1 were investigated in female rats because these enzymes have been implicated in ET-743 metabolism (21). Levels of the latter two were dramatically decreased to almost unmeasurable levels 3 days after administration and returned to control values within 12–24 days. The activity of CYP3A2 was decreased by up to 37% at day 3 after administration, and this decrease persisted for up to 12 days. Levels of total microsomal cytochrome P450 protein were diminished by 33% after 3 days.

**Effect of ET-743 on Hepatic Gene Expression.** Gene expression profiles were analyzed using one microarray for each of the three pairs of female rats per time point. A cluster of genes was consistently down-regulated, and this cluster included the cytochrome P450 genes Cyp1a2, Cyp3a11, and Cyp3a13, mirroring the down-regulation in activity of CYP1A1/2, CYP3A2, and CYP2E1 (see Fig. 3), and Car3, which codes for carbonic anhydrase 3. Car3 expression levels reached a nadir on day 6 after administration of ET-743 (data not shown), past the time point of maximal biochemical manifestation of hepatic damage, which was day 3. Among the cluster of overexpressed genes were the cell cycle genes Cdc2a and Ccnd1 (Fig. 4A); the rodent homologues of human CDC2 and cyclin D1, respectively; and the two ABC transport genes Abcb1a and Abcb1b, which are equivalent to human ABCB1 and impart drug resistance. The time course of expression of Abcb1a and Abcb1b (Fig. 5) mirrors the changes in serum bilirubin.
HEPATOXICITY OF ET-743

received ET-743 was significantly elevated in comparison with that in control rats (Fig. 1, H and I, and Fig. 4B). Similar to the results shown here for Ki-67 (Fig. 1, H and I), staining for proliferating cell nuclear antigen was substantially elevated in ET-743-treated rats 3 days after dosing compared with control animals (data not shown). The weight of livers of ET-743-treated animals increased from day 2 after dosing onward and reached a zenith on day 12 (Fig. 4B). Furthermore, consistent with an ET-743-mediated increase in hepatocyte cycle activity, liver cell cycle distribution underwent significant, albeit subtle, alteration by ET-743. The proportion of $S$-phase hepatocytes increased from 0% in control rats to $7.3 \pm 1.5\%$ (mean $\pm$ SD; $n = 4$) in rats 3 days after administration of ET-743, whereas the number of hepatocytes in $G_1$ and $G_2$-$M$, respectively, amounted to $62.6 \pm 7.2\%$ and $37.4 \pm 7.2\%$ in control animals and $61.0 \pm 5.2\%$ and $31.4 \pm 5.3\%$ in treated rats.

Effect of ET-743 on Indices of Hepatic Cell Proliferation. The increase in hepatic expression of $Cdc2a$ determined by DNA microarray peaked on day 3 after administration of ET-743. It remained elevated through at least day 6 and returned to basal levels on day 24 (Fig. 4A). $Ccd1$ expression followed a similar pattern, except that the elevation was smaller and maintained through day 24 (Fig. 4A). The $Cdc2a$ gene expression changes were similar to ET-743-induced changes in hepatic Ki-67 proliferation index. The peak in Ki-67 proliferation and $Cdc2a$ expression occurred at 6 days and 9 days, respectively, before the peak in liver weight (Fig. 4B). Staining for Ki-67, a marker of DNA synthesis, in liver nuclei of animals that had concentration (Fig. 3). The expression of none of the other 45 ABC genes among the expressed sequence tags on the microarray was altered by ET-743. ABC genes unaffected by ET-743 include $Abcb1a$, $Abcb1b$, $Abcb4$, and $Abcg2$. Furthermore, consistent with an ET-743-mediated increase in hepatocyte cycle activity, liver cell cycle distribution underwent significant, albeit subtle, alteration by ET-743. The proportion of $S$-phase hepatocytes increased from 0% in control rats to $7.3 \pm 1.5\%$ (mean $\pm$ SD; $n = 4$) in rats 3 days after administration of ET-743, whereas the number of hepatocytes in $G_1$ and $G_2$-$M$, respectively, amounted to $62.6 \pm 7.2\%$ and $37.4 \pm 7.2\%$ in control animals and $61.0 \pm 5.2\%$ and $31.4 \pm 5.3\%$ in treated rats.

DISCUSSION

Various cancer chemotherapeutic agents are known to possess the potential to damage the liver (22). Methotrexate is a prominent example, which has been reported to cause steatosis and cirrhosis in patients (23). The results described above suggest that the novel anticancer drug ET-743 induces an unusual form of hepatotoxicity in rats, which differs from the adverse hepatic effects described for traditional cytotoxic drugs. In the female rat, a clinically relevant single i.v. dose of 40 $\mu$g/kg ET-743 caused damage to bile duct epithelia followed by peribiliary fibrosis. Bile duct damage accompanied by inflammation and repair was first evident 24 h after dosing. In contrast, cytotoxic damage by ET-743 to the rapidly proliferating cells of the small intestine was observed by 6 h. Fibrosis around damaged bile ducts increased dramatically over the following week. The pathological alterations were accompanied by defects of liver function, as reflected by a dramatic elevation of plasma bilirubin levels, moderate increases in plasma levels of ALP and AST, a marker of cholestasis, and a decrease in activities of hepatic cytochrome P450 enzymes CYP3A2, CYP1A1/2, and CYP2E1. These observations are consistent with the notion that ET-743 induces biliary cholestasis, which in turn might elicit elevation in expression of the $Abcb1a$ and $Abcb1b$ genes as an adaptive response. This interpretation is based on the previous observation that expression of these genes was raised in cholestatic livers of rats and monkeys (24).

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In analogy, the down-
regulation of expression of the Car 3 gene described above may also be the consequence of hepatobiliary injury rather than a direct response to ET-743 proper because it has been shown to occur in the livers of rodents exposed to the hepatotoxicant griseofulvin.6

It is unknown whether the reduction in CYP3A2, CYP1A1/2, and CYP2E1 levels observed here in the rat also occurs in humans. If it did, it might be important in the planning of sequential combination chemotherapy involving ET-743 and drugs such as Taxol, which undergo cytochrome P450-mediated deactivation. The decrease in CYP activities, if it occurs in humans, probably does not impact the pharmacokinetics of the parent drug because ET-743 pharmacokinetics were unaltered when the drug was administered repeatedly via 24-h infusion (6).

Experiments in which ET-743 was incubated with isolated hepatocytes from rats or humans suggest that concentrations of the drug as high as 0.01–1 nmol/ml are required to damage hepatocytes directly (data not shown). In a preliminary study, hepatic drug levels in rats 6 h after i.v. administration of ET-743 (40 μg/kg) were just above 1 pmol/g tissue and declined thereafter.7 These results render it unlikely that concentrations of ET-743 required to elicit toxicity in isolated liver cells in vitro were achieved in liver tissue for time periods sufficient to explain the damage observed after administration of ET-743 in vivo. Instead, it is probable that manifestation of ET-743-mediated hepatic damage in the rat requires the structural integrity of the whole liver. This conclusion is consistent with the fact that the primary and most important lesion was found in the bile ducts. It is conceivable that drug accumulates in the bile duct, thus precipitating the primary lesion. This hypothesis needs experimental validation.

A prominent feature of the ET-743-induced hepatic toxicity described here is the persistence of the damage. Bile duct fibrosis and increased plasma levels of AST were observed as late as 3 months after administration, and levels of ALP were elevated for up to 2 months. In contrast, bilirubin remained elevated until some time between days 12 and 24. It remains to be investigated whether the long-term persistence of liver damage induced by ET-743 is related to continued harmful levels of a ET-743 metabolite in the liver. Nevertheless, it appears more likely that these delayed manifestations are late consequences of the initial bile duct damage and ensuing fibrosis. It is important to note that in contrast to the long persistence of elevation of AST and ALP in the female rat, transaminitis in patients is sensitive than female animals to the adverse effects of the drug. This finding suggests that at comparable doses, male rats are less responsive than female animals to ET-743-induced hepatotoxicity are also highlighted by the fact that the dose required to elicit damage in livers of male rats was twice as high as that which caused hepatic toxicity in female animals. This finding suggests that at comparable doses, male rats are less sensitive than female animals to the adverse effects of the drug. Nevertheless, qualitatively similar changes in pathology and biochemical marker levels were induced by the drug in animals of either gender. Concentrations of ET-743 that precipitate toxicity in the liver are at yet unknown. Therefore, it is conceivable that the observed gender difference in susceptibility reflects differences in liver exposure to ET-743, perhaps as a consequence of gender-related discrepancies in ability to metabolize the drug.

The most striking result of the DNA microarray analysis of hepatic gene expression precipitated by ET-743 is the increased expression of the cell cycle genes Cdc2a and Ccnd1. The time course of change in expression of the Cdc2a gene resembles the time course of the ET-743-induced alterations in liver weight and Ki-67 labeling. These observations suggest that ET-743 elicits a mitogenic wave in the liver by induction of DNA synthesis, a proposition that is further supported by augmented staining of hepatic nuclei for proliferating cell nuclear antigen, the increased proportion of liver cells in S phase, and the prominent presence of mitotic figures in livers of animals that had received ET-743. To our knowledge, such stimulation of mitogenesis has hitherto not been observed as a generic mechanistic feature of hepatotoxic drugs. Therefore, it is unlikely to be a nonspecific compensatory reaction of the tissue to the ET-743-induced lesion. Instead the mitogenic wave seems to be a ET-743-specific phenomenon, and it is probably the corollary of a direct effect of ET-743 on hepatic Cdc2a transcription, which in turn drives the cell cycle. Whether or not patients exposed to ET-743 experience a similar mitogenic wave and increase in liver mass is not known and should be monitored in future studies.

In conclusion, the toxicity exerted by ET-743 in the rat liver is characterized by a primary insult to the bile duct epithelium, long duration of altered liver pathology, and enhanced liver cell proliferation involving up-regulation of the Cdc2a and Ccnd1 genes. It has to be stressed that the animal model chosen here to study ET-743 hepatotoxicity, the female rat, seems among all species hitherto investigated to be most exquisitely sensitive to the hepatotoxic potential of ET-743. Other species including humans are less likely to experience the adverse effects described here. Nevertheless, manifestations of hepatotoxicity have been observed in the clinical evaluation of the drug, and therefore it is conceivable that some of the features of toxicity delineated here in the female rat are applicable to patients. These features may provide the basis for the design of treatment regimens designed to reduce the hepatotoxic potential of ET-743.

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