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ORIGINAL ARTICLE

**Sarah Donald · Richard D. Verschoyle
Peter Greaves · Samantha Orr · Jose Jimeno
Andreas J. Gescher**

Comparison of four modulators of drug metabolism as protectants against the hepatotoxicity of the novel antitumor drug yondelis (ET-743) in the female rat and in hepatocytes in vitro

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Abstract *Purpose*: Yondelis (ET-743), a tetrahydroisoquinoline alkaloid isolated from a marine tunicate, is a novel drug with demonstrated anticancer activity in early clinical trials against sarcoma, breast and ovarian carcinoma. Yondelis has myelotoxic and hepatotoxic side effects, the latter reflected by reversible transaminitis and cholangitis. In the female rat pretreatment with high-dose dexamethasone has been shown to abrogate yondelis-mediated hepatotoxicity, an effect tentatively linked to its ability to induce cytochrome P450 CYP3A isoenzymes, which metabolize yondelis. Here we tested the hypothesis that pretreatment of rats with modulators of hepatic drug metabolism, β-naphthoflavone, phenobarbitone or N-acetylcysteine, protect rat livers against the effects of yondelis. *Methods*: Female rats received yondelis (40 μg/kg intravenously) and liver damage in vivo was assessed in terms of changes in plasma levels of bilirubin, alkaline phosphatase (ALP) and aspartate aminotransferase (AST) and by histopathology. In order to investigate yondelis toxicity in vitro, hepatocytes isolated from untreated rats or from rats pretreated with dexamethasone, β-naphthoflavone or phenobarbitone were maintained in culture and exposed to yondelis. *Results*: Pretreatment with β-naphthoflavone and phenobarbitone ameliorated yondelis-mediated hepatotoxicity in vivo. The former abrogated plasma indicators on day 3, but hardly on day 6, and the latter suppressed elevation of bilirubin, but not of ALP or AST. Pretreatment with N-acetylcysteine did not protect from, but slightly exacerbated, yondelis-induced liver changes. Hepatocytes from naive animals or from pretreated rats did not differ in their susceptibility towards yondelis-induced cytotoxicity in vitro. Nor did inclusion of N-acetylcysteine (1 m*M*) in the cellular incubation medium affect yondelis-induced hepatocytotoxicity. *Conclusions*: The results suggest that certain inducers of cytochrome P450 enzymes such as dexamethasone and β-naphthoflavone can protect rat liver against the unwanted effects of yondelis, but such protection cannot be mimicked in in vitro experiments using liver cells in culture.

Keywords Dexamethasone · ET-743 · Hepatotoxicity
β-Naphthoflavone · Phenobarbitone

Abbreviations *ALP* Alkaline phosphatase · *ANIT* α-Naphthylisothiocyanate · *AST* Aspartate aminotransferase · *DMSO* Dimethylsulfoxide · *LDH* Lactate dehydrogenase

S. Donald · R. D. Verschoyle · A. J. Gescher (✉)
Department of Oncology,
University of Leicester, LRI, 5th floor RKCSB,
Leicester, LE2 7LX, UK
E-mail: ag15@le.ac.uk
Tel.: +44-116-2231856
Fax: +44-116-2231855

P. Greaves
Medical Research Council Toxicology Unit,
University of Leicester,
Leicester, LE1 9HN, UK

S. Orr
UK Human Tissue Bank,
De Montfort University,
Leicester, LE1 5XY, UK

J. Jimeno
PharmaMar SA, 28770 Colmenar Viejo,
Madrid, Spain

Introduction

Yondelis (ecteinascidin 743, ET-743) is a tetrahydroisoquinoline alkaloid isolated from the marine tunicate *Ecteinascidia turbinata* (for structure see Fig. 1). It possesses potent antineoplastic activity against a variety of human tumor xenografts grown in athymic mice, including melanoma and ovarian and breast carcinoma [1, 2, 3]. In clinical phase I studies of yondelis, promising responses were observed in patients with sarcoma and breast and ovarian carcinoma. Therefore this new drug is currently under intensive investigation in several phase

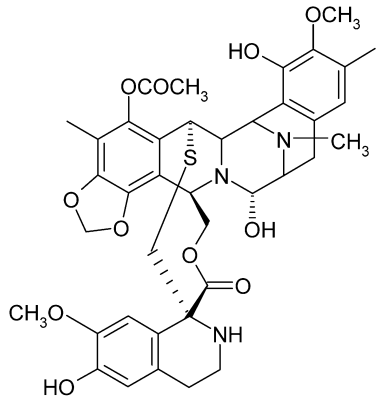


Fig. 1 Structure of yondelis

II clinical trials in cancer patients with a variety of neoplastic diseases [4, 5]. Yondelis has myelotoxic and hepatotoxic side effects [6]. Patients who have received yondelis by prolonged infusion over 24–72 h experience myelosuppression and, frequently, acute, albeit reversible, elevation of transaminases and subclinical cholangitis characterized by increases in alkaline phosphatase (ALP) and/or bilirubin [7, 8].

Preclinical acute toxicity studies conducted in mice, rats, dogs and monkeys have consistently demonstrated liver toxicity as an important side effect of yondelis, as evidenced by an increase in plasma levels of liver-specific enzymes and pathological manifestations of cholangitis. Recently the nature and extent of the hepatobiliary changes exerted by yondelis in the female rat, the species which is most susceptible to the hepatotoxic potential of yondelis, have been characterized by histopathology, electron microscopy, immunohistochemistry, plasma biochemistry and DNA microarray analysis [9]. Furthermore, pretreatment with high-dose dexamethasone has been shown to abrogate yondelis-mediated hepatotoxicity in this animal model without impeding its antitumor activity [10]. Protection by dexamethasone pretreatment is accompanied by a dramatic reduction in hepatic levels of yondelis, tentatively implicating elevated hepatic clearance of yondelis, perhaps via induced metabolic enzymes, as the mechanism by which dexamethasone exerts its beneficial effect.

In the work described here, the observations pertinent to the hepatotoxicity of yondelis in the female rat were extended. The hypothesis was tested that β-naphthoflavone, phenobarbitone and N-acetylcysteine, three well-characterized modulators of drug metabolism, protect the liver against yondelis. Their hepatoprotective capacities were compared with that of dexamethasone. β-Naphthoflavone, phenobarbitone and dexamethasone induce cytochrome P450 enzyme families CYP1A1/2, CYP2B and CYP3A, respectively, and thus can increase the rate of oxidative metabolic disposition of suitable drug substrates. N-Acetylcysteine replenishes intrahepatocellular stores of thiol moieties and increases levels of glutathione, thus buttressing the capacity of the cell to detoxify chemically reactive, potentially harmful drug-derived species [11].

In general, the capacity of drugs to elicit liver damage in vivo is often the result of a series of complex cellular processes that involve the hepatic uptake of the drug, its biotransformation and its elimination from the liver. Most of the current understanding of liver damage induced by xenobiotics has been obtained in rodent models, and sometimes this information predicts toxicity in humans. In the light of the current trend to reduce animal experimentation, liver cells from rodents or humans in suspension or culture are often used to screen drugs for hepatotoxic potential and to reveal insights into mechanisms by which they may exert such toxicity. However, it is important to explore for each new drug, to what extent experiments using hepatocytes in vitro can reliably predict potential hepatotoxicity which may occur in vivo. Mindful of these considerations we tested the hypothesis that protective activity exerted by dexamethasone, β-naphthoflavone, phenobarbitone or N-acetylcysteine against yondelis-induced hepatic changes in vivo can be observed in vitro in cultured hepatocytes isolated from rats which have undergone appropriate pretreatment. Finally, we assessed in a preliminary fashion the similarity between the responses to yondelis of liver cells from rats and of those from a human. Overall the aim of the study was to define early in the drug development process experimental approaches which may help to characterize protection measures against the adverse hepatic effects of this promising new anticancer agent.

Materials and methods

Animals and materials

Wistar rats were obtained from Charles River Laboratories (Margate, UK). Pure yondelis and yondelis for injection formulation were provided by the drug manufacturer PharmaMar (Colmenar Viejo, Madrid, Spain). The injectable yondelis formulation used in the in vivo experiments was identical to that employed in the clinical evaluation of the drug. In the hepatocyte cultures pure substance was added after dissolution in DMSO. All other materials were purchased from Sigma-Aldrich (Poole, UK). The animal experiments were conducted as stipulated by Project Licences 80/1250 and 40/2496 granted by the UK Home Office. The experimental design, which complied with the UKCCCR guidelines for the welfare of animals in experimental neoplasia, was vetted and approved by the Leicester University Ethical Committee for Animal Experimentation.

Study of hepatotoxicity in vivo

Female Wistar rats (230–260 g) were pretreated with metabolism modulators and then received yondelis at 40 μg/kg (or 240 μg/m^2) intravenously via the lateral tail vein. This dose was chosen because it had been found previously [9] to be an "optimal" hepatotoxic dose, in that whilst it constitutes about half of the published maximum tolerated dose in the female rat when given as a single intravenous dose [6], it invariably elicits hepatotoxicity. In comparison, clinical doses which have been used range from 600 to 1800 μg/m^2 given as infusions over periods of 1 to 24 h [6], a common regimen employing 1500 μg/m^2 as a 24-h infusion. The choice of dose of modulator was based on relevant literature

(quoted below), which defines optimal metabolizing enzyme-inducing (dexamethasone, β-naphthoflavone, phenobarbitone) or electrophile-scavenging activity (N-acetylcysteine) doses. Treatment was as follows: dexamethasone, single dose of 20 mg/kg, dissolved in glycerol formal, orally 24 h prior to yondelis [11]; β-naphthoflavone, three doses of 75 mg/kg orally daily on three consecutive days, with the last dose given 48 h prior to yondelis [12]; phenobarbitone 500 mg/l of drinking water for 7 days prior to yondelis [12]; and N-acetylcysteine, single dose of 200 mg/kg, intraperitoneally 1 h before yondelis [13]. Control animals received the vehicle only, i.e. glycerol formal in the case of dexamethasone and β-naphthoflavone, and water for yondelis. Each treatment group comprised four animals. Hepatic changes were assessed in terms of alterations in plasma levels of bilirubin and liver enzymes ALP and aspartate aminotransferase (AST) and by histopathological investigation of liver tissue, as described previously [9].

Hepatocyte isolation and incubation

Rats were treated with dexamethasone (three daily doses of 50 mg/kg /day orally), β-naphthoflavone or phenobarbitone (doses as described above). Rats were killed 24 h after the final dose of dexamethasone, β-naphthoflavone or phenobarbitone (see above). Hepatocytes were isolated by the two-stage in situ perfusion technique described by Seglen [14]. Initial cell viability was 85% or higher, as determined by trypan blue exclusion.

Cell suspensions were diluted to 1×10^6 cells/ml of culture medium (Williams E medium containing 2 m*M* glutamine, 2 m*M* gentamicin, 10 n*M* insulin, 5 μ*M* transferrin, 5 m*M* nicotinamide, 0.75 μg/ml zinc sulfate, 0.2 μg/ml copper sulfate, 5 ng/ml sodium selenite, 30 n*M* dexamethasone sodium phosphate, 10% fetal calf serum, penicillin/streptomycin 100U/100 μg/ml) and plated (2×10^6 per well) onto fibronectin-coated six-well plates and incubated at 37°C in an atmosphere of 5% CO_2 and 95% air for 4 h to allow attachment to the substratum. The supernatant medium was removed and fresh medium was added. Human hepatocytes were obtained from the UK Human Tissue Bank, De Montfort University, Leicester. Isolated human hepatocytes were cultured as described for rat hepatocytes. Hepatocytes were precultured for 24 h, then exposed to fresh medium, and yondelis dissolved in DMSO was added to cultures to yield final concentrations in the range 1 n*M* to 10 μ*M*. The final DMSO concentration was 1%, which on its own did not affect hepatocyte viability.

For investigation of the effect of N-acetylcysteine on the hepatocytotoxicity of yondelis, hepatocytes were exposed to N-acetylcysteine (1 m*M*), and yondelis was added 1 h later. In an orientation experiment this N-acetylcysteine concentration was found to increase intracellular non-protein thiol levels within 1 h by 100% (result not shown). Hepatocytotoxicity was assessed by leakage of lactate dehydrogenase (LDH) into the medium. Samples of medium were taken after 48, 72 and 96 h for viability assessment by the LDH assay kit (Sigma-Aldrich, Poole, UK). The degree of leakage of LDH into the medium in the sample culture was expressed as a percentage (x) in relation to the total cellular LDH enzyme content. Total LDH content was established by adding Triton X-100 to untreated hepatocyte cultures, which caused complete lysis. Viability was calculated as $100-x$, and expressed as a percentage.

Results

Effects of β-naphthoflavone, phenobarbitone and N-acetylcysteine on yondelis-induced plasma indicators of hepatotoxicity

Rats were pretreated with β-naphthoflavone, phenobarbitone or N-acetylcysteine before they received yondelis. The consequence of pretreatment with these agents for yondelis-mediated hepatic alterations as indicated by levels of bilirubin, ALP and AST in the plasma are demonstrated in Fig. 2. Yondelis administered on its own elicited dramatically raised biochemical indicators in the plasma. These changes were maximal on day 3 after administration of yondelis, and still detectable on day 12 [9]. Pretreatment with β-naphthoflavone with the last of three doses given 24 h prior to yondelis ameliorated the yondelis-induced liver changes in that the elevation of all three indicators of hepatic damage observed on day 3 was abrogated. The protective effect lasted in part to day 12, in that levels of AST and bilirubin in rats pretreated with β-naphthoflavone were indistinguishable from those seen in control, i.e. untreated, rats, yet ALP levels were elevated in the range seen in rats which had received yondelis only. Addition of phenobarbitone to the drinking water for 1 week prior to administration of yondelis had no significant effect on the rise in ALP and AST activities elicited by yondelis. However, it prevented yondelis-induced elevation of plasma bilirubin levels. Pretreatment with N-acetylcysteine had no protective effect, as levels of bilirubin, AST and ALP were as elevated as those in animals which received yondelis only. As described before, dexamethasone given 24 h prior to yondelis completely abolished the yondelis-induced rise in plasma bilirubin, ALP and AST for at least 12 days [10].

Effects of β-naphthoflavone, phenobarbitone and N-acetylcysteine on yondelis-induced histopathological changes in liver

Histopathological examination underpins the biochemical results described above (Fig. 3). Livers of animals which had received yondelis alone revealed degenerative and inflammatory changes in the biliary epithelium (Fig. 3B) compared to livers of control rats (Fig. 3A). Pretreatment with β-naphthoflavone abolished the degenerative and inflammatory effects on the biliary epithelium on day 3 (Fig. 3D). At 12 days peribiliary fibrosis was less marked in these rats than in those which had received yondelis only. In contrast, β-naphthoflavone treatment appeared to have little or no impact on the degree of hepatocellular necrosis induced by yondelis at 12 days. In rats pretreated with phenobarbitone inflammatory changes in the bile ducts and lymphoid depletion were marginally less pronounced than in rats which had received yondelis only, but differences were slight. Rats which had received N-acetylcysteine followed by yondelis displayed significantly more biliary damage than rats treated with yondelis alone (Fig. 3C). In one animal on the combination, focal areas of bile duct epithelium showed complete necrosis. Nevertheless, there was no evidence of any increase in the extent of hepatocellular necrosis in these animals. Consistent with a previous report [10], hepatic alterations were abrogated in rats which had received dexamethasone before yondelis (Fig. 3E).

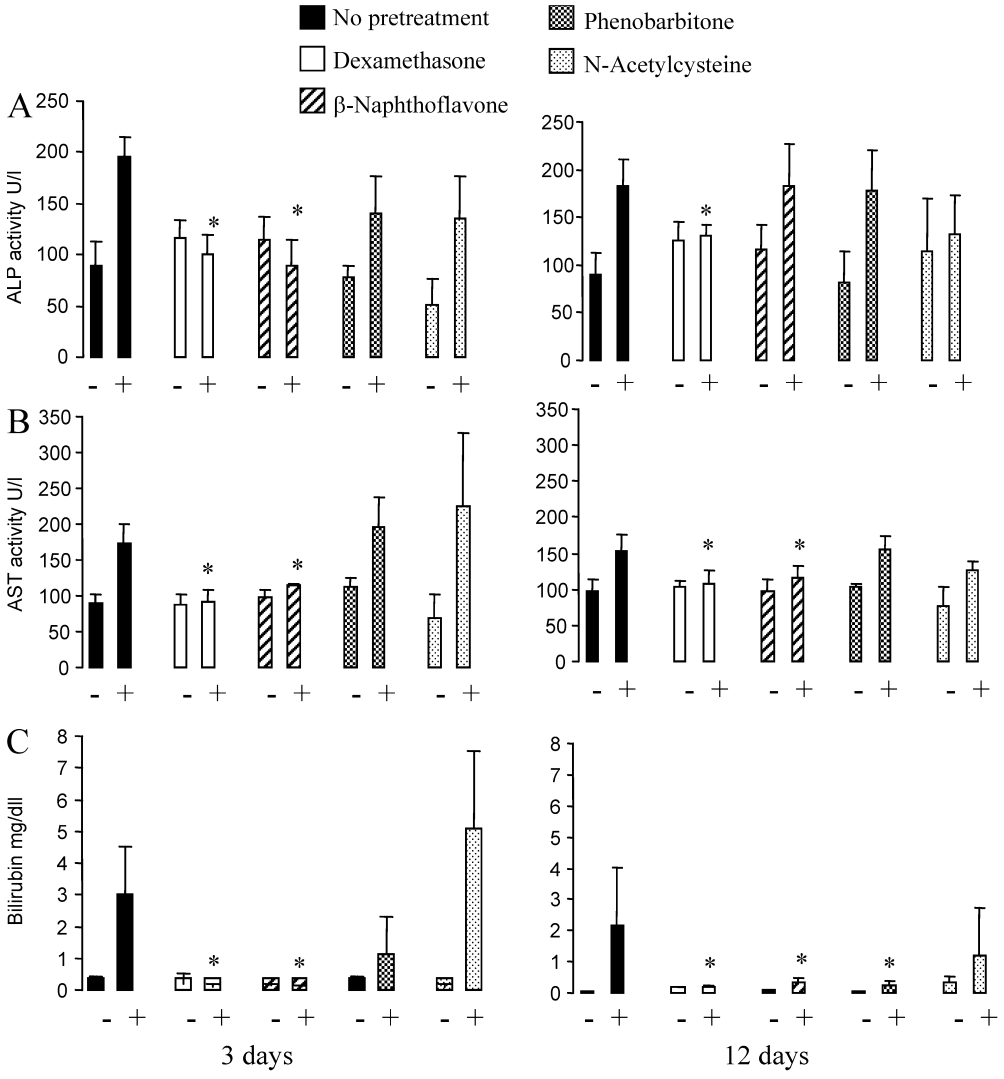


Fig. 2A–C The effect of dexamethasone, *β*-naphthoflavone, phenobarbitone and N-acetylcysteine administered prior to yondelis (40 μg/kg, intravenous) on yondelis-induced elevation of plasma activities of the liver-specific enzymes ALP (**A**), AST (**B**) and of plasma levels of total bilirubin (**C**) in female Wistar rats. Indicators were measured 3 and 12 days after yondelis administration (+ rats treated with yondelis, – animals treated with yondelis vehicle water; *filled bars* animals without pretreatment, *open bars* animals pretreated with dexamethasone 20 mg/kg orally 24 h prior to yondelis, *striped bars* animals pretreated with three doses of *β*-naphthoflavone 75 mg/kg orally 72, 48 and 24 h prior to yondelis, *chequered bars* animals pretreated with phenobarbitone 500 mg/l of drinking water for 7 days prior to yondelis, *stippled bars* animals pretreated with N-acetylcysteine 200 mg/kg intraperitoneally 1 h prior to yondelis). For details of animals and treatments see Materials and methods. Values are the means ± SD of four animals. *$P < 0.01$ vs yondelis only, by ANOVA

Effects of pretreatment with dexamethasone, *β*-naphthoflavone, phenobarbitone and N-acetylcysteine on yondelis-induced cytotoxicity in hepatocytes in vitro

Incubation of rat hepatocytes in culture with yondelis resulted in a time- and dose-dependent decrease in cell viability. Viabilities of hepatocytes after a 48-h exposure to yondelis at 1, 10, 100 and 1000 n*M* were 77 ± 3%, 65 ± 7%, 53 ± 2% and 46 ± 7%, respectively, compared to control (untreated) hepatocytes. After 96 h, hepatocyte viabilities were 64 ± 2%, 26 ± 3%, 19 ± 4% and 5 ± 1%, respectively. Exposure of human hepatocytes to 100 n*M* yondelis resulted in a time-dependent decrease in cell viability which was comparable to, but slightly less severe than, that seen in cultures of rat hepatocytes (Fig. 4). This result may suggest that under the experimental conditions used here, human hepatocytes are less susceptible than rat hepatocytes towards the direct cytotoxic potential of yondelis.

Next the effect of pretreatment of rats with *β*-naphthoflavone, dexamethasone or phenobarbitone, and of coincubation of hepatocytes with N-acetylcysteine, on the cytotoxicity of yondelis (in the range 1 n*M* to 1 μ*M*) was explored. Figure 5 shows that the viability of liver cells from control (un-pretreated) rats and rats which had been pretreated with dexamethasone, *β*-naphthoflavone or phenobarbitone were equally susceptible to the toxic potential of yondelis, as reflected by LDH release. Likewise, inclusion of N-acetylcysteine (1 m*M*) in cultures of hepatocytes from naive rats did not alter their LDH release characteristics in response to exposure to yondelis.

Discussion

The work described above provides essentially two major novel insights into the hepatotoxicity of yondelis in the rat, which permit conclusions germane to potential hepatoprotective strategies to be drawn: (1) pretreatment with *β*-naphthoflavone and phenobarbitone afforded some protection against the detrimental effect of yondelis on rat liver; and (2) none of the pretreatment

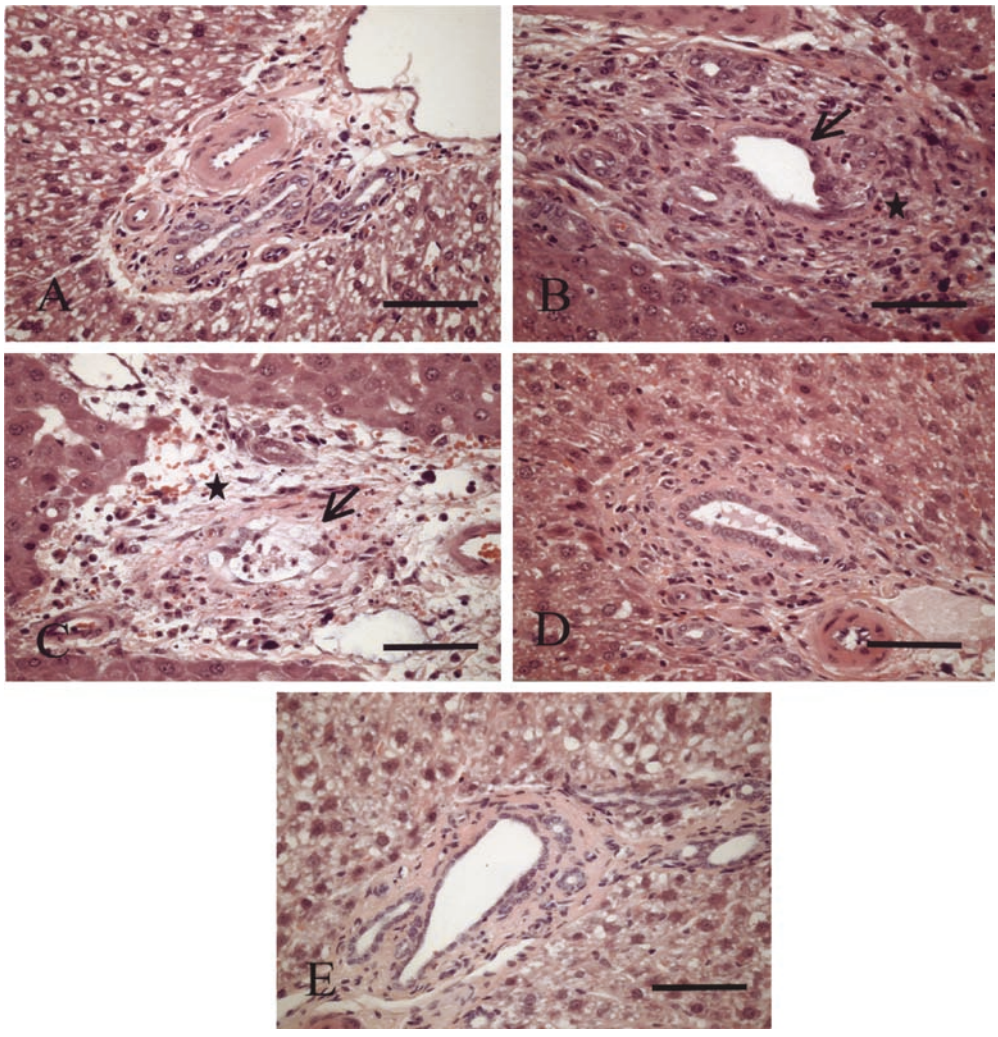


Fig. 3A–E The effect of N-acetylcysteine, β-naphthoflavone and dexamethasone on yondelis-induced changes in liver pathology. Pictures show liver sections from a control (vehicle-treated) female Wistar rat (**A**) and from animals that received yondelis alone (40 μg/kg intravenously, **B**) or yondelis 1 h (**C**) or 24 h (**D**, **E**) after either N-acetylcysteine (single dose of 200 mg/kg intraperitoneally, **C**), or the last of three doses of β-naphthoflavone (75 mg/kg orally on three consecutive days, **D**) or dexamethasone (single dose of 20 mg/kg orally, **E**). Liver tissue was excised 3 days after administration of yondelis, at which time yondelis-inflicted damage was maximal [9]. Staining was by hematoxylin and eosin. Note in **B** the enlarged, thickened portal tract with a sparse infiltrate of inflammatory cells (*star*) and the damaged bile duct (*arrow*), which is lined by degenerative and reactive epithelium, characteristics of yondelis-induced changes in livers of female rats. These features are somewhat exacerbated by N-acetylcysteine (**C**) as indicated by the almost total necrosis of the bile duct epithelium (*arrow*) and the surrounding swelling and cell debris (*star*) in the portal tract. In contrast, in livers of rats pretreated with β-naphthoflavone (**D**) or dexamethasone (**E**), comparable portal tracts are almost indistinguishable from those of controls. Sections are representative of four to eight separate animals. For details on treatment and histopathology see Materials and methods (*bars* 100 μm)

strategies demonstrated to be protective in vivo affected the cytotoxicity exerted by yondelis in hepatocytes in vitro. The protection provided by β-naphthoflavone and phenobarbitone in vivo was not as efficacious as that afforded by dexamethasone. Amelioration of yondelis-mediated hepatotoxicity by β-naphthoflavone persisted only for a short time, and that by phenobarbitone was weak, as reflected by significant suppression of elevation of only one biochemical indicator, bilirubin, but not of the others. Another agent, N-acetylcysteine, failed to protect rat livers against yondelis altogether. These findings complement our recent detailed characterization of dexamethasone as a potent antidote against yondelis-mediated hepatotoxicity in the female rat [10]. The protection afforded by dexamethasone was accompanied by dramatically decreased hepatic levels of yondelis and by upregulated CYP3A enzyme levels, suggesting that protection by dexamethasone is the corollary of the increased clearance of the drug from the liver, possibly mediated by metabolism involving CYP3A [10].

Information on the metabolism of yondelis is sparse and inconclusive. How can the findings presented here be integrated with what is currently known about yondelis metabolism catalyzed by CYP enzymes? In studies using rat liver microsomes in vitro, the metabolism of yondelis as reflected by its disappearance from the incubation medium is impeded by inhibitors of

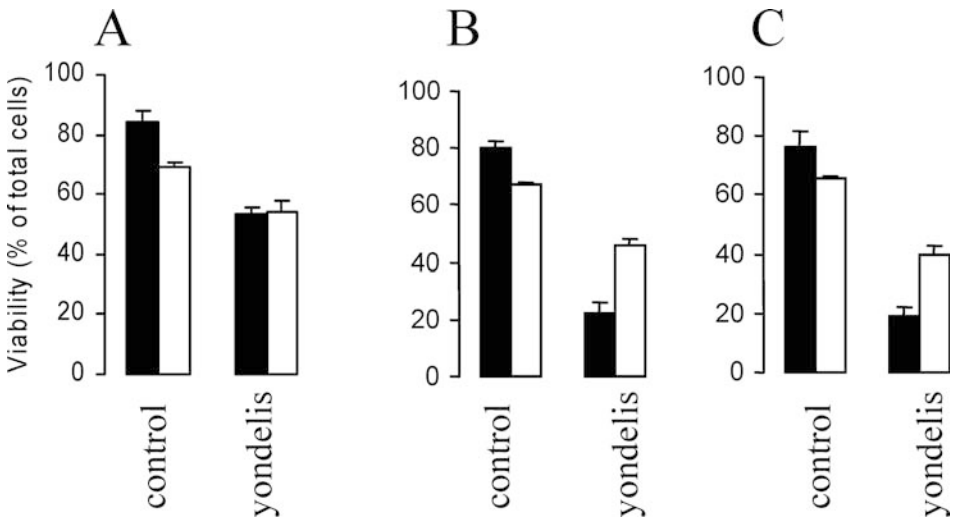


Fig. 4A–C Effect of yondelis on viability of hepatocytes from rats (*closed bars*) and humans (*open bars*) measured after incubation with the drug for 48 h (**A**), 72 h (**B**) or 96 h (**C**). Hepatocytes in culture were exposed to yondelis (100 n*M*) or the vehicle (DMSO, control). Hepatocyte viability was assessed by measurement of LDH in the cellular supernatant. Viability was calculated by expressing LDH release in the culture as percentage (x) of the value obtained in untreated cultures in which all cells were destroyed to cause maximal LDH release, and by subtracting x from 100. For details of hepatocyte isolation, culture and viability measurement see Materials and methods. Values are the means ± SD of three separate experiments

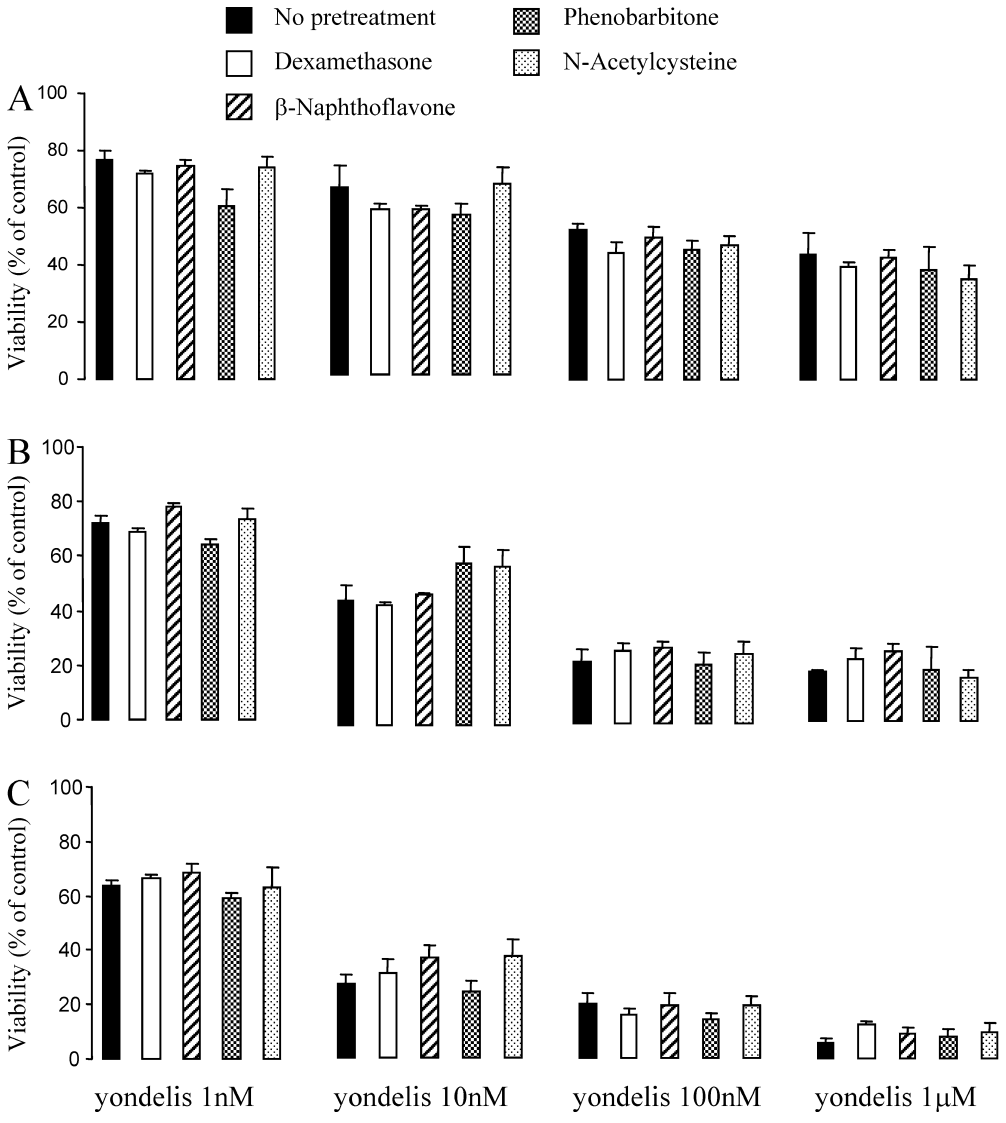
CYP3A, CYP2C and CYP1A enzymes [15, 16]. The metabolism of yondelis is enhanced in incubations of liver microsomes from rats which had been treated with dexamethasone and phenobarbitone, inducers of CYP3A and CYP2B, respectively, when compared to microsomes from untreated rats, whilst yondelis metabolism in microsomes from rats which had received methylcholanthrene, an inducer of CYP1A, is indistinguishable from that in controls [16]. In a study in which yondelis was incubated with lymphoblasts expressing human CYP enzymes, CYP3A4, CYP2D6, CYP2E1 and CYP2C9 seemed to catalyze its disappearance, whilst CYP1A2, CYP1B, CYP2B6, CYP2C8 or CYP2C19 did not [17]. CYP3A is known to be expressed much more in male than in female rats.

A key observation in support of a major role of CYP3A enzymes in yondelis metabolism is the finding that liver microsomes form male rats metabolize the disappearance of yondelis to a greater extent than those from females [16]. On the basis of these findings, tentative inferences can be made as to the mechanisms by which β-naphthoflavone and phenobarbitone ameliorated the effects of yondelis on the rat liver, as outlined in the results described here. β-Naphthoflavone is an inducer of CYP1A, phenobarbitone induces several CYPs, especially CYP2B, in addition to phase II metabolizing enzymes [18]. Raised CYP enzyme levels as a consequence of pretreatment with β-naphthoflavone or phenobarbitone may have been, at least in part, responsible for their hepatoprotective efficacy. It is important to note though that these agents possess a variety of physiological effects which may contribute to hepatoprotection. In the case of phenobarbitone, it is well known that it induces liver bile flow [19], and this property may have been involved with the protection afforded by phenobarbitone.

The untoward hepatic effects of many hepatotoxicants, for example N-methylformamide [20], which comprise alkylating or arylating moieties, or which generate such functionalities via metabolism, can efficiently be counteracted by strategies leading to the elevation of intracellular thiols. Conversely, other agents can undergo metabolic toxification by reaction with glutathione. An instructive example is α-naphthylisothiocyanate (ANIT), which produces cholestasis, hyperbilirubinemia, and bile duct epithelial cell necrosis

►

Fig. 5A–C Effect of yondelis on viability of hepatocytes isolated from rats which have been pretreated with dexamethasone, β-naphthoflavone or phenobarbitone, or of cells which have been incubated with N-acetylcysteine (*closed bars* hepatocytes from naive animals, *open bars* hepatocytes from rats which treated with dexamethasone 20 mg/kg orally 24 h prior to killing, *striped bars* hepatocytes from animals treated with three doses of β-naphthoflavone 75 mg/kg orally 72, 48 and 24 h prior to killing, *chequered bars* hepatocytes from animals treated with phenobarbitone 500 mg/l of drinking water for 7 days prior to hepatocyte isolation, *stippled bars* hepatocytes from naive rats in culture including 1 m*M* N-acetylcysteine from 1 h prior to addition of yondelis onwards). Hepatocyte viability was assessed at 48 h (**A**), 72 h (**B**) and 96 h (**C**) after commencement of yondelis treatment by measurement of LDH in the cellular supernatant. Values are percentages with respect to the viability of hepatocytes at the respective time-point in culture omitting yondelis (= 100%) and are the means ± SD of three separate experiments. The viabilities of hepatocytes cultured without yondelis with respect to initial viability (= 100%) were as follows: at 48 h 82 ± 3% (control), 80 ± 2% (dexamethasone), 83 ± 2% (β-naphthoflavone), 80 ± 1% (phenobarbitone) and 81 ± 3% (N-acetylcysteine); at 72 h 77 ± 3% (control), 74 ± 1% (dexamethasone), 76 ± 2% (β-naphthoflavone), 75 ± 1% (phenobarbitone) and 76 ± 3% (N-acetylcysteine); at 96 h 68 ± 3% (control), 68 ± 3% (dexamethasone), 67 ± 4% (β-naphthoflavone), 66 ± 4% (phenobarbitone) and 69 ± 3% (N-acetylcysteine). For details of hepatocyte isolation, culture and viability measurement see Materials and methods



in rats, changes which are not dissimilar to those induced by yondelis. ANIT undergoes metabolic reaction with glutathione to generate a dithiocarbamate intermediate [21]. The conjugate is thought to be transported into the bile, where it can revert back to glutathione and ANIT, resulting in the accumulation of ANIT at cytotoxic concentrations in the biliary tract. The finding that N-acetylcysteine failed to ameliorate yondelis-induced hepatotoxicity militates against the possibility that the hepatic alterations consequent upon yondelis treatment were caused by reactive electrophiles which escape efficient detoxification by rapid reaction with intrahepatocellular thiols. Consistent with this interpretation, additional experiments demonstrated that yondelis at the hepatotoxic dose used in this study failed to deplete hepatic non-protein thiols in rats (result not shown). It needs to be stressed that yondelis levels in the rat liver after the hepatotoxic dose of 40 μg/kg are unlikely to confound hepatic thiol status, with peak levels of yondelis in the liver unlikely to exceed 40 pmol/g tissue [10].

Therefore, if yondelis reacts covalently with non-protein thiols at all, it is likely that hepatic thiol stores suffice for efficient metabolic transformation. All these findings are consistent with the notion that hepatic glutathione does not detoxify yondelis. There was even an indication of intensification of the toxicity of yondelis, especially on histopathological observation, elicited by pretreatment with N-acetylcysteine. This finding hints at the possibility that yondelis reacts with non-protein thiols to yield a hepatotoxic species, or a precursor thereof, in analogy to the mechanism by which ANIT is thought to cause hepatotoxicity. Nevertheless, as the exacerbation of yondelis-induced hepatic changes by N-acetylcysteine was not profound, this inference has to be drawn with utmost caution and requires further experimental verification.

Pretreatment with dexamethasone, *β*-naphthoflavone or phenobarbitone at doses which decreased the hepatotoxic potential of yondelis in the female rat in vivo failed to influence cytotoxicity exerted by yondelis in hepatocytes in vitro. This result suggests that hepato-

cytes in culture are unlikely to be a suitable model to aid with the discovery of agents which protect against the detrimental hepatic effects of yondelis. Moreover it reinforces the deductions drawn from histopathological observations [9], which suggest that yondelis causes damage in the rat initially to bile duct epithelia, eliciting inflammation followed by peribiliary fibrosis and then hepatic necrosis. Thus it is probable that manifestation of yondelis-induced hepatic damage in the rat requires the structural integrity of the whole liver, and that yondelis accumulates in the bile duct, thus giving rise to the primary lesion.

In conclusion, cultured hepatocytes do not provide a suitable model for studying the hepatotoxicity of yondelis. Therefore strategies designed to eliminate yondelis-mediated hepatotoxicity need to be tested in vivo. Alternatively it is conceivable that sophisticated experimental designs involving high-precision liver slices or hepatocytes and bile cells in co-culture may model in vitro hepatic changes mediated by yondelis in the rat in vivo. Of all the potential protectants tested by us thus far dexamethasone and β-naphthoflavone have been most interesting, the former displaying high, the latter moderate, efficacy as potential antidotes against yondelis-induced hepatotoxicity. It is conceivable that induction of enzymes which catalyze the oxidative metabolism of yondelis is a mechanism which curtails its hepatotoxic potential.

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