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Complete Protection by High-Dose Dexamethasone against the Hepatotoxicity of the Novel Antitumor Drug Yondelis (ET-743) in the Rat[1]

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ABSTRACT

Yondelis (ET-743) is a promising antitumor drug with hepatotoxic properties in animals and humans. Here the hypothesis was tested that dexamethasone can ameliorate manifestations of yondelis-induced hepatotoxicity in the female Wistar rat, which is the animal species with the highest sensitivity toward the adverse hepatic effect of yondelis. Hepatotoxicity was adjudged by measurement of plasma levels of alkaline phosphatase, aspartate aminotransferase, and bilirubin, and by liver histopathology. Yondelis (40 μg/kg i.v.) alone caused a dramatic elevation of plasma alkaline phosphatase, aspartate aminotransferase, and bilirubin levels, and degeneration and patchy focal necrosis of bile duct epithelial cells. Pretreatment of rats with dexamethasone (5–20 mg/kg, p.o.) 24 h before yondelis ameliorated or abrogated the biochemical and histopathological manifestations of yondelis-induced liver changes. In contrast, when dexamethasone was administered simultaneously with yondelis, its toxicity was not reduced. Pretreatment with dexamethasone (10 mg/kg) also reversed the gene expression changes induced by yondelis in rat liver. However, dexamethasone pretreatment did not interfere with the antitumor efficacy of yondelis in rats bearing the 13762 mammary carcinoma or in four murine models. Dexamethasone (10 mg/kg) administered 24 h before yondelis decreased hepatic levels of yondelis dramatically compared with those obtained after administration of yondelis alone, whereas yondelis plasma levels after the drug combination were not markedly different from those in rats on yondelis alone. The results suggest that pretreatment with high-dose dexamethasone effectively protects rats against yondelis-mediated hepatic damage by decreasing hepatic exposure to yondelis, perhaps linked to induction of metabolism by cytochrome P450 enzymes. Pretreatment with high-dose dexamethasone should be investigated in patients who receive yondelis to ameliorate its unwanted effect on the liver.

INTRODUCTION

The history of the development of novel anticancer drugs provides ample examples of molecules that, despite impressive antineoplastic activity in preclinical models, struggled to reach the stage of routine clinical application because of severe host toxicity. Among these molecules are cisplatin, camptothecin, and paclitaxel, the development of which was hampered by their nephrotoxic, bladder toxic, and neutropenic/neurotoxic properties, respectively. Therefore, the search for strategies to ameliorate or abrogate adverse drug effects is increasingly an important component of the early clinical development of novel anticancer drugs. The new experimental drug yondelis (ecteinascidin-743, ET-743), a tetrahydroisoquinoline alkaloid isolated from the marine tunicate *Ecteinascidia turbinata*, 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 yondelis, promising responses were observed in patients with sarcoma, breast, and ovarian carcinoma (4, 5), and the drug is currently under intense investigation in a variety of Phase II trials in cancer patients. As for many anticancer drugs, the dose-limiting toxicity of yondelis in patients is myelotoxicity. Another toxicological Achilles heel of yondelis is its hepatotoxic potential as reflected by an increase in plasma levels of liver-specific enzymes and pathological manifestations of cholangitis in most animal species in which it has been tested (6). Patients who received yondelis by prolonged infusion over 24–72 h experienced myelosuppression and, frequently, acute, albeit reversible, elevation of transaminases and subclinical cholangitis characterized by increases in ALP[3] and/or bilirubin. The mechanisms by which yondelis causes liver damage are unclear, and an initial deleterious attack by yondelis on the bile duct seems important (7). The mechanisms by which yondelis causes antitumor activity involve profound perturbations of the cell cycle subsequent to interaction of the drug with DNA. In contrast to most alkylating agents, which bind guanine at positions N7 or O6 in the major groove of DNA, yondelis binds guanine at N2 in the minor groove, at preferred sequences such as 5′-PuGC and 5′-PyGG (8). This and probably related interactions enable yondelis to inhibit the binding to DNA of sequence-specific factors involved in transcription activation, exemplified by its ability to block the transcription of heat shock protein 70 induced by temperature elevation (9) and *multidrug resistance 1* gene induced by exposure to a histone deacetylase inhibitor (10). Significantly, both of these genes are under the control of NF-Y. Intriguingly, yondelis is more cytotoxic against cells that are proficient in nucleotide excision repair than against nucleotide excision repair-deficient cells, which are characterized by hypersensitivity to UV rays, cisplatin, and conventional alkylating agents (11, 12). None of the mechanistic elements germane to antitumor activity have thus far been associated with the etiology of yondelis-induced hepatotoxicity.

We wished to explore experimental strategies aimed at the amelioration of drug-induced hepatotoxicity to aid the additional clinical development of this promising anticancer drug. On the basis of preliminary clinical observations (13), we reasoned that the steroid dexamethasone might afford protection against yondelis-induced hepatotoxicity. This hypothesis was tested in female rats, the animal species that has been shown to be most susceptible toward the adverse hepatic side effect of yondelis (6, 7). Furthermore, the effect on antitumor activity of combining yondelis with dexamethasone was explored in five rodent models. To gain insight into the mechanism by which dexamethasone may affect yondelis-mediated hepatotoxicity,

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[3] The abbreviations used are: ALP, alkaline phosphatase; AST, aspartate aminotransferase; TW, tumor weight; EST, expressed sequence tag; HPLC, high-performance liquid chromatography; AUC, area under the plasma concentration-*versus*-time curve.

Table 1 *Activity of dexamethasone, ET-743 and the combination in mouse B-16 melanoma, M5076 reticulum cell sarcoma, IGROV-1 ovarian carcinoma and TE-671 rhabdomyosarcoma*

	Dose schedule		TWI (%)[1]		
Tumor model	Dexamethasone[2]	ET-743[3]	Dexamethasone	ET-743	Combination
B-16[4]	40 mg/kg on days 7, 8, 9, 14, 15, 16[8]	0.15 mg/kg on days 10, 17	52**	45**	69**,⇓
M5076[5]	40 mg/kg on days 11, 12, 13, 18, 19, 20	0.15 mg/kg on days 14, 21	10	64**	65**
IGROV-I[6]	40 mg/kg on days 8, 9, 10, 15, 16, 17, 22, 23, 24	0.2 mg/kg on days 11, 18, 25	41	56**	60**
TE-671[7]	20 mg/kg on days 12, 13, 14, 15	0.2 mg/kg on day 15	15	33*	68**,⇓

[1] TWI = tumor weight inhibition = 100 − (T/Cx100), with T = weight of treated animals, C = weight of control animals, for measurement of tumor weight see Materials and Methods
[2] via the *i.p.* route
[3] via the *i.v.* route
[4] Tumor weight evaluated on day 23, control tumors weighed 6.13 ± 0.59 g (mean ± S.E.).
[5] Tumor weight evaluated on day 32, control tumors weighed 2.89 ± 0.25 g (mean ± S.E.).
[6] Tumor weight evaluated on day 32, control tumors weighed 3.84 ± 1.11 g (mean ± S.E.).
[7] Tumor weight evaluated on day 34, control tumors weighed 4.90 ± 0.22 g (mean ± S.E.).
[8] Days post tumor implantation.
* Tumor weight significantly different from untreated mice ($p < 0.05$, Fisher's test).
** Tumor weight significantly different from untreated mice ($p < 0.01$, Fisher's test).
⇓ Tumor wieght significantly different from ET-743-treated mice ($p < 0.05$, Fisher's test).

we measured levels of yondelis in the liver and plasma of rats pretreated with dexamethasone.

MATERIALS AND METHODS

Animal and Agents. Wistar rats (used in the studies of hepatotoxicity, gene expression changes, hepatic CYP3A function, and yondelis plasma levels), Fischer rats, and mice of the C57Bl/6J, MCr-*ν*/*ν*, and CDI *ν*/*ν* strains (used in the antitumor activity studies) were obtained from Charles River Laboratories (Margate, United Kingdom or Rome, Italy). Yondelis formulated for injection originated from the drug manufacturer PharmaMar SA (Colmenar Viejo, Spain). The specific CYP3A substrate 7-benzoyloxyquinolone and its metabolite 7-hydroxyquinolone were obtained from BD Biosciences (Oxford, United Kingdom), all other reagents from Sigma-Aldrich Co. Ltd. (Poole, United Kingdom).

Study of Hepatotoxicity. Female Wistar rats (230–260 g) were pretreated with a single dose of dexamethasone (5–20 mg/kg, dissolved in glycerol formal, p.o.; Ref. 14) 24 h before, or simultaneous with, a hepatotoxic dose of yondelis (40 μg/kg, i.v. via the lateral tail vein). Control animals received the vehicles used, *i.e.*, glycerol formal in the case of dexamethasone, and water as the vehicle for yondelis. Each treatment group comprised 5 animals. Hepatic changes were studied by assessment of alterations in plasma levels of bilirubin and liver enzymes ALP and AST, and by conventional histopathological investigation of liver tissue as described previously (7). Experiments were conducted as stipulated by Project License 80/1250 granted by the United Kingdom Home Office, and the experimental design was vetted and approved by the Leicester University Ethical Committee for Animal Experimentation.

Study of Antitumor Activity. The 13762 tumor was propagated in female Fischer rats. Tumor fragments (100–200 mg weight) were implanted (s.c.) into the flank of rats (100–120 g). Female C57Bl/6J mice (20 ± 2 g body weight) received 10^6 B16F1 melanoma cells s.c. or 10^5 M5076 reticulum cell sarcoma cells i.m. Female MCr-*ν*/*ν* mice (22 ± 2 g body weight) received 5×10^6 Igrov/1 ovarian carcinoma cells s.c. Male CDI *ν*/*ν* mice (20 ± 2 g body weight) received 1.5×10^6 TE-671 rhabdomyosarcoma cells s.c. Properties of the tumor models have been described in the following representative references: 13762 (15), B16F1 (16), M5076 (17), Igrov/1 (18), and TE 671 (19). Rats (8/group) received dexamethasone (10 mg/kg, i.p.) on day 9 after 13762 tumor implantation. Yondelis (30 or 40 μg/kg, i.v.) was administered 24 h after dexamethasone. Thus, the treatment protocol in the rat tumor model mimics faithfully the protocol used in the hepatoprotection studies. In the murine models mice (10/group) received repeated doses of dexamethasone (20 or 40 mg/kg i.p.) daily between days 7 and 24 after tumor implantation. Yondelis (0.15 or 0.2 mg/kg, i.v.) was administered once, twice, or three times between days 10 and 25 after tumor implantation. The specific dosing schedules used in each model are listed in the legend to Table 1. TW was determined on days 14 and 17 after tumor implantation in rats, and between days 23 and 34 after tumor implantation in mice, as detailed for each model in Table 1. TW was calculated via tumor diameter, using a Vernier caliper and the formula

$$\text{TW (in mg)} = \text{tumor volume (m)}^3 = d^2 \times D/2$$

in which d and D represent the shortest and longest diameter, respectively. Procedures involving animal care and treatment were conducted as stipulated in Italian National Guidelines (D.L. No. 116 G.U., suppl. 40, 18.2.1992, circolare No. 8, G.U. luglio 1994) and appropriate European directives (EEC Council Directive 86/609, 1.12.1987), and adhered to the Guide for the Care and Use of Laboratory Animals (United States National Research Council, 1996).

cDNA Microarray Study. For the analysis of differential gene expression, control rats were paired with rats that had received yondelis (40 μg/kg, i.v.), dexamethasone (10 mg/kg, p.o.), or the combination of dexamethasone and yondelis, the latter given 24 h afterdexamethasone. Hepatic gene expression was analyzed using a cDNA microarray containing 4700 hybridizable ESTs. Analysis of hepatic gene expression was carried out 3 days after administration of yondelis using cDNA microarrays containing ~4700 hybridizable mouse ESTs derived from IMAGE clones obtained from Research Genetics (Huntsville, AL) or from the Medical Research Council Human Gene Mapping Project.[4] One array was used for each pair of rats with the individual RNAs being labeled with Cy3 or Cy5. The labels were reversed for subsequent hybridizations. Microarray preparation, RNA labeling, and hybridization were performed as described previously (7, 20).

Study of CYP3A Function. Livers of control and dexamethasone-pretreated rats were homogenized in Tris-KCl buffer (50 mM) containing sucrose (0.25 M; pH 7.4). For isolation of liver microsomes the homogenate was centrifuged (10,000 × *g*, 20 min, 4°C), and the supernatant was then removed and spun at 100,000 × *g* for 60 min at 4°C. The microsomal pellet was resuspended in fresh buffer and recentrifuged (100,000 × *g*) for an additional60 min. The resulting pellet was suspended in 0.25 M phosphate buffer containing 30% glycerol, stored at −80°C, and thawed before analysis. Microsomal protein concentration was determined with the Bradford assay. The ability of the microsomes to metabolize the CYP3A model substrate 7-benzoyloxyquinolone to 7-hydroxyquinolone was tested as described by Renwick *et al.* (21).

Study of ET-743 Levels in Liver and Plasma. Blood samples and liver tissue were collected from rats before and up to 72 h after administration of yondelis (40 μg/kg i.v.) with or without pretreatment with dexamethasone (10 mg/kg p.o.) given 24 h before yondelis. Blood samples were taken by cardiac puncture and placed in heparinized tubes. Plasma was obtained by centrifugation, and an aliquot (0.3 ml) was mixed with 0.7 ml ammonium acetate buffer (0.2 M; pH 5.0). The mixture was kept on ice (30 min), centrifuged (1000 × *g*, 10 min) and subjected to solid phase extraction (Bond Elut nonend-capped CN cartridge). Methanolic hydrochloric acid (0.1 M, 2.5 ml) served as eluant. The elution solvent was evaporated, the residue dissolved in

[4] Internet address: http://www.le.ac.uk/mrctox/microarray_lab/.

mobile phase (200 μl), and an aliquot (10 μl) was injected onto the HPLC column. Liver tissue was homogenized (1:1) in water. An aliquot (0.5 ml) of the homogenate was extracted with 10 parts of hydrochloric acid (0.1 M) in methanol. After shaking for 30 min, samples were centrifuged (1500 × *g*, 4°C, 10 min). The supernatant was dried under nitrogen, and the residue was dissolved in mobile phase (200 μl, acetic acid 0.1% in water:acetonitrile 90:10). Reconstituted liver extract was filtered through a PTFE filter (Alltech, Deerfield, IL), and an aliquot (10 μl) was injected onto the HPLC column. ET-729 served as internal standard in all of the analytical assays, and in orientation experiments ET-729 was not detected as a metabolite of yondelis in any of the tissues studied. Yondelis was measured by HPLC coupled to electrospray ionization tandem mass spectrometry, as described by Rosing *et al.* (22) using an API 3000 triple quadrupole mass-spectrometer (Applied Biosystems-Sciex, Toronto, Ontario, Canada) operating in positive ion mode (standard TurboIonSpray source). Separation were achieved on a Luna C_{18} column (Phenomenex, Torrance, CA), and a mobile phase of acetic acid (0.1%) in water (solvent A) and acetonitrile (solvent B), with gradient elution of B from 10 to 70% in 10 min, at the flow rate of 200 μl/min. Calibration curves of standard solutions of yondelis (0.05–25 ng/g) were prepared using plasma or liver homogenate from untreated mice. The correlation between concentration and peak response in these extracts analyzed on three different days was characterized by $r^2 > 0.999$. Quality control samples of plasma (spiked with 0.1, 0.5, and 2 ng yondelis/ml) or liver homogenate (from livers spiked with 1, 5, and 20 ng yondelis/g tissue) afforded limits of quantitation of 0.05 ng/ml for plasma and 0.5 ng/g for liver; the precision between runs was reflected by correlation coefficients of ±5% (plasma) and ±10% (liver), and the accuracy was in the range of 97–105% (plasma) and 90–110% (liver).

The AUC for yondelis was calculated by the trapezoideal rule, between time point 0 and 6 h, the last time point at which yondelis was quantifiable in the plasma.

RESULTS

Effect of Pretreatment with Dexamethasone on Yondelis-induced Liver Changes. Yondelis administered on its own elicited hepatic damage as reflected by dramatically raised bilirubin, and elevated ALP and AST in the plasma (Fig. 1). Histological examination of the livers from these rats showed that from day 3 after administration onwards portal tracts were swollen and contained a sparse acute inflammatory infiltrate. Epithelial cells lining bile ducts showed focal degeneration and mild hyperplasia. Round zones of hemorrhagic hepatocellular necrosis were also present. All of these changes are identical with the yondelis-induced liver alterations in female Wistar rats reported earlier (7). A single dose of dexamethasone (5–20 mg/kg) administered alone caused loss of up to 10% body weight within a day of administration, but animals recovered that loss subsequently. Rats that received dexamethasone did not exhibit significant liver changes, except for slight hepatic vacuolation, conceivably because of lipid, and clear cell change, probably caused by glycogen. Dexamethasone (5–20 mg/kg) given 24 h before yondelis abolished the yondelis-induced rise in plasma indicators of hepatic damage (Fig. 1). The results reported in Fig. 1 were obtained on day 3 after yondelis, the time point at which liver damage was maximal in rats that received yondelis alone (7). Pathological examination of the liver supported these biochemical observations. Dexamethasone 5 mg/kg reduced the severity of the bile duct damage generated by yondelis in all of the rats, and 1 animal was completely damage-free. None of these animals showed hemorrhagic necrosis. At 20 mg/kg dexamethasone completely abrogated the histopathological changes elicited by yondelis, and at 10 mg/kg histopathological changes were minimal (Fig. 2). The protective efficacy of dexamethasone was validated in livers of rats obtained at two additional time points, 6 and 12 days after administration of yondelis. As observed on day 3, dexamethasone (10 mg/kg) abrogated the biochemical changes and strongly attenuated the histopathological manifestations in the liver elicited by yondelis. These experiments were repeated using dexamethasone (5–20 mg/kg) in a 3-daily repeated dose schedule, which proved also protective, but resulted in greater glucocorticoid-related weight loss than that associated with a single dose.

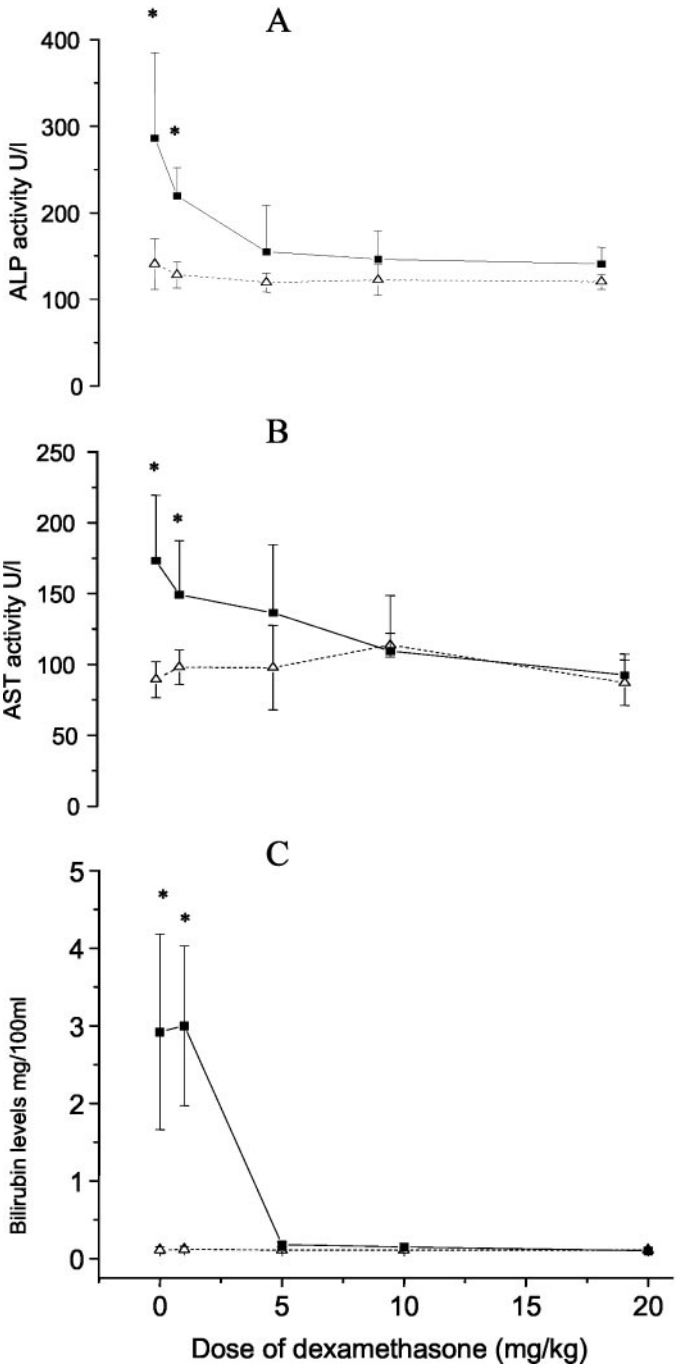

Fig. 1. Dose dependency of the effect of dexamethasone administered p.o. 24 h before yondelis (40 μg/kg, i.v.) on yondelis-induced elevation of plasma activities of the liver-specific enzymes ALP (*A*), AST (*B*), and of plasma levels of total bilirubin (*C*) in female Wistar rats measured 3 days after yondelis. △ reflect values observed in rats that received either dexamethasone alone or glycerol formal, the vehicle for dexamethasone (0 mg/kg dose of dexamethasone), ■ denote values in rats which received the combination and animals that received yondelis alone (0 mg/kg dose of dexamethasone). For details of experimental design see "Materials and Methods." Values are the mean ± SD of 5 animals. Stars indicate that values are significantly different ($P < 0.01$ by ANOVA) from activities and levels in animals that did not receive yondelis.

To assess whether dexamethasone-mediated hepatoprotection is rat strain-dependent or affected by the presence of tumors, its effect on the hepatotoxicity of yondelis in female Fischer rats bearing the 13762 tumor was investigated. The degree of liver changes induced by

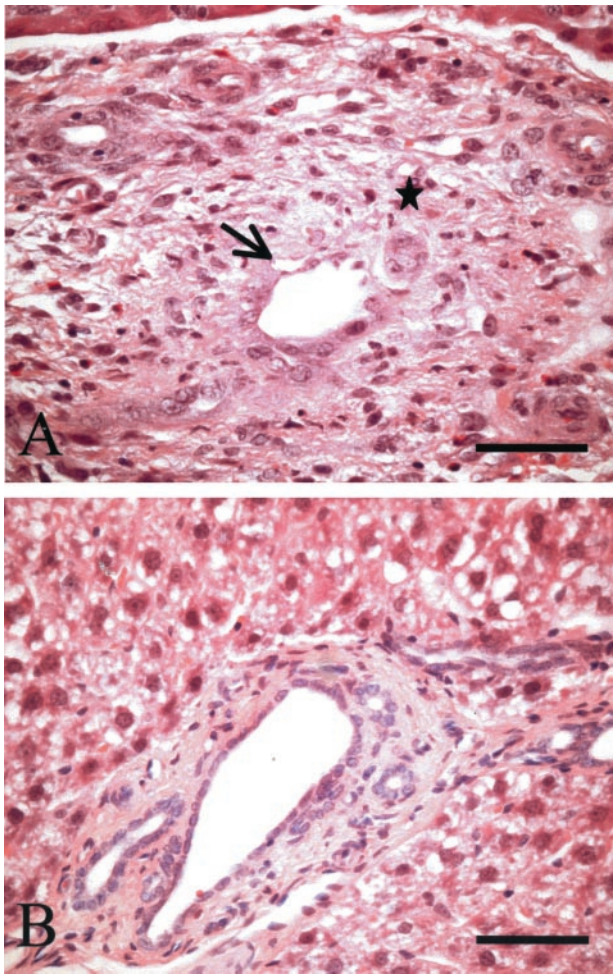

Fig. 2. Liver sections from female Wistar rats which received yondelis (40 μ/kg i.v.) alone (*A*) or 24 h after dexamethasone (10 mg/kg, p.o.; *B*). Liver tissue was excised 3 days after administration of yondelis, at which time yondelis-inflicted damage was maximal (8). Staining was by H&E. Note in *A* the swollen, 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. In contrast, after dexamethasone (*B*) a comparable portal tract is indistinguishable from controls. Sections are representative of four to eight separate animals. For details on treatment and histopathology see "Materials and Methods." *Bar* equals 100 μm.

yondelis alone, as adjudged by plasma levels of liver-specific indicators and liver histopathology, and the protective efficacy of pretreatment with dexamethasone (10 mg/kg) given 1 day before yondelis, were indistinguishable from those described above for Wistar rats.

Effect of Simultaneous Treatment with Dexamethasone on Yondelis-induced Liver Changes. Dexamethasone in the 0.01–0.03 mg/kg dose range is often administered in the clinic at the same time as cytotoxic drugs to prevent drug-induced emesis. Therefore, coadministration of yondelis with high-dose dexamethasone as a potential hepatoprotectant and antiemetic would clearly be clinically expedient. However, when coadministered with yondelis, dexamethasone (10 mg/kg) failed to protect rat livers against yondelis toxicity. In animals that received the combination, plasma ALP and bilirubin levels were as elevated as those seen in rats on yondelis only. Moreover, plasma AST activity in rats on the combination was 4-fold higher than that measured in rats treated with yondelis alone; AST activities (in units per liter) were 85 $\pm$ 12 in control rats, 155 $\pm$ 19 in rats on yondelis alone, and 615 $\pm$ 116 in rats on the combination (mean $\pm$ SD; $n = 4$). Likewise, simultaneous dosing of dexamethasone with yondelis failed to abrogate the histopathological changes induced by yondelis alone. Swelling of the portal tract was greater in 2 of 4 rats that received the combination, and the livers of these rats showed many more foci of hemorrhagic hepatocellular necrosis than did livers of rats on yondelis alone.

Effect of Pretreatment with Dexamethasone on Antitumor Activity of Yondelis. To justify exploration of the dexamethasone hepatoprotection strategy in the clinic, it is of paramount importance to demonstrate that dexamethasone does not adversely affect the antitumor activity of yondelis. This hypothesis was tested initially in female Fischer rats bearing the 13762 mammary carcinoma. Fig. 3 shows that both dexamethasone (10 mg/kg, i.p.) and yondelis (40 μg/kg, i.v.) individually exhibited activity in this model, and that the combination was not inferior to that of the single agents. In the case of tumor evaluation on day 14, the percentage TW inhibition of the combination was 68 or 36% higher than the values observed with yondelis or dexamethasone, respectively, alone. Very similar results as those shown in Fig. 3 were obtained when the dose of yondelis was reduced to 30 μg/kg. To explore the generic nature of the antitumor results, we investigated the activity of the combination of yondelis with dexamethasone pretreatment also in murine tumor models of melanoma (B-16), reticulum cell sarcoma (M 5076), ovarian carcinoma (IGROV-1), and rhabdomyosarcoma (TE-671). A variety of dosing regimes, and dose levels of yondelis and dexamethasone was used to account for differential sensitivities of the individual models and to allow discrimination between efficacies of the individual constituents (Table 1). Dexamethasone exhibited significant antineoplastic activity on its own in the murine B16 melanoma and IGROV-1 ovarian tumor models. In none of the rodent models investigated was the combination less efficacious than yondelis administered on its own. Moreover, in three of the models investigated, the 13762 (Fig. 2), B16, and TE-671 models (Table 1), the combination was clearly more efficacious than yondelis alone.

Effect of Pretreatment with Dexamethasone on Changes in Hepatic Gene Expression Caused by Yondelis. We reported previously that the hepatotoxicity of yondelis is accompanied by changes in

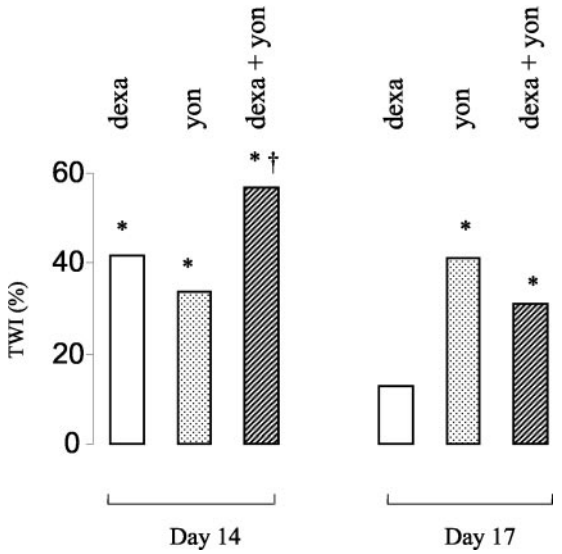

Fig. 3. Antitumor activity of dexamethasone (*dexa*, 10 mg/kg i.p.), yondelis (*yon*, 40 mg/kg i.v.) or the combination (*dexa+yon*), with dexamethasone given 24 h before yondelis, in female Fischer rats bearing the 13762 mammary carcinoma. For details of tumor implantation, treatment and measurement see "Materials and Methods." Values are expressed as TW inhibition [TWI = 100 − (T/C×100), with T = weight of treated animals, C = weight of control animals]. TW in control rats was 1.60 $\pm$ 0.27 g (mean $\pm$ SE), *star* indicates that TW values were significantly different ($P < 0.01$ by Fisher's test) from controls, *dagger* indicates that TW in animals which received the combination was significantly different from that in rats on yondelis only ($P < 0.05$ by Fisher's test).

the expression of a variety of hepatic genes (7). To assess whether dexamethasone at a hepatoprotective dose abrogates these changes, hepatic gene expression was analyzed in livers obtained 3 days after administration of yondelis using a cDNA microarray containing 4700 hybridizable ESTs as described before (7, 20). For the analysis of differential gene expression, each rat treated with yondelis, dexamethasone, or the combination was paired with a control rat. Fig. 4 shows the 25 most abundantly expressed hepatic genes induced by yondelis alone *versus* their expression pattern in animals that received yondelis after pretreatment with dexamethasone. Among the genes up-regulated by yondelis alone were, as demonstrated previously (7), the cell cycle genes *cdc2a* and *ccnd1*, the rodent homologues of human cdc2 and cyclin D1, and the two drug-resistance coding ABC transport genes *abcb1a* and *abcb1b*. Up-regulation of the cell cycle genes was probably a direct effect of yondelis, whereas increased expression of the transport genes was possibly a response to the drug-induced damage (7). The expression of the genes that were up-regulated by yondelis was reduced to control levels by pretreatment with dexamethasone, with the exception of expression of the genes coding for metalloproteinases 1 and 2, which remained elevated (Fig. 4). When gene expression was compared under identical conditions between animals that received dexamethasone alone and those that were treated with the combination, a pattern comparable with that shown in Fig. 4 was observed.

Treatment with yondelis alone also resulted in the down-regulation of several genes, including the cytochrome P450 genes *cyp1a1*, *cyp2e1*, and *cyp3a25*. Pretreatment with dexamethasone abolished the decrease in expression of these genes caused by yondelis alone (data not shown). It also ameliorated the yondelis-induced down-regulation of expression of the hemoglobin genes *hbb-b1*, *hbb-y*, *hba-a1*, and *hba-x*, but did not abolish it totally. Overall, the results of the microarray study support the notion that dexamethasone pretreatment ameliorates the gene expression changes preceding or accompanying the histopathological manifestations and plasma indicators of the detrimental effect of yondelis on the rat liver.

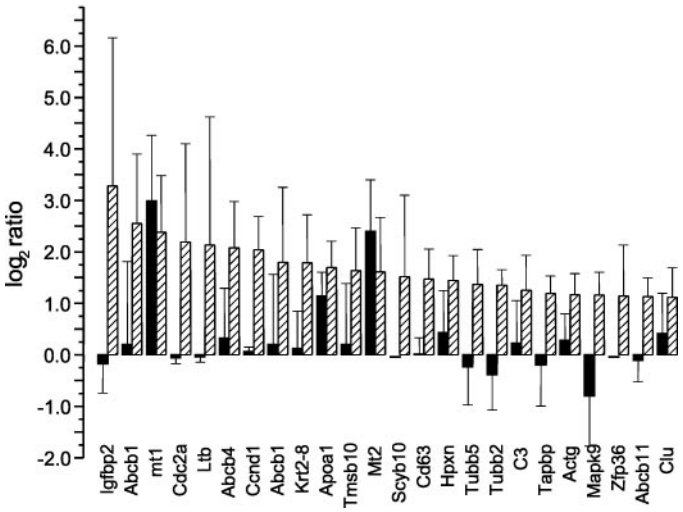

Fig. 4. Gene expression changes elicited by yondelis without pretreatment (▨) and after pretreatment with dexamethasone (■) in livers of female Wistar rats. Values are expressed as log mean ratio of fold expression; *bars*, ± SD. Rats received dexamethasone (10 mg/kg p.o.) 24 h before yondelis (40 μg/kg, i.v.). Gene expression was analyzed in livers obtained 3 days after treatment with yondelis, which is the time point of maximal toxicity (7). Rats treated with yondelis or with the combination of yondelis with dexamethasone were each paired with control (untreated) rats. Three pairs of rats were used for each comparison. Differentially expressed genes were identified using a two-tailed *t* test of the mean ratio of fold gene expression against the population mean. Differential expression was considered significant for genes characterized by *P*s of <0.05. Plotted for each gene are the log2 ratios, ± SD, of the change in expression of treated *versus* untreated rats. The genes shown are ordered by decreasing expression ratio as determined in the analysis of yondelis treatment *versus* control. Gene symbols are shown below expression ratios, and gene names are as follows: Igfbp2, insulin growth factor binding protein 2; Abcb1, ATP binding cassette protein B1 (two different ESTs were used); mt1, metallothionein 1; Cdc2a, cell division cycle 2 (cdk1); Ltb, lymphotoxin B; Abcb4, ATP binding cassette protein B4; Ccnd1, cyclin D1; Krt2–8, keratin complex 2, basic, gene8; Apoa1, apolipoprotein A-1; Tmsb10, thymosin β; Mt2, metallothionein 2; Scyb10, small inducible cytokine B10; Cd63, CD63 antigen; Hpxn, hemopexin; Tubb5, tubulin β 5; Tubb2, tubulin β 2; C3, complement component 3; Tapbp, TAP binding protein; Actg, actin γ cytoplasmic; Mapk9, mitogen activated protein kinase 9; Zfp36. zinc finger protein 36; Abcb11, ATP binding cassette protein B11; and Clu, clusterin. For additional details of treatment and microarray analysis see "Materials and Methods."

Effect of Dexamethasone on Hepatic Cytochrome P450 3A Enzymes. In a series of additional experiments biochemical changes in the liver elicited by dexamethasone, which may contribute to the mechanism by which it protects the rat liver against yondelis-inflicted damage, were explored. Hepatic gene expression was analyzed using cDNA microarray and the ESTs described above. Livers were obtained from pairs of untreated rats and rats that had received dexamethasone (10 mg/kg) 1 day before RNA isolation, so that the analysis was performed exactly at the time point at which yondelis would have been injected in the experiments in which the combination was studied. Dexamethasone up-regulated the expression of eight genes by 2.5-fold or more, and these included genes involved in fatty acid synthesis, gluconeogenesis, and carbohydrate metabolism. Most dramatic induction was observed for the cytochrome P450 genes *cyp3a11* (23.2-fold induction), *cyp3a16* (18.1-fold), *cyp3a25* (13.2-fold), and *cyp3a13* (10.2-fold). To validate at the functional level the induction of CYP3A gene expression by dexamethasone, liver microsomes of dexamethasone-pretreated rats were obtained, and their ability to metabolize the model substrate 7-benzoyloxyquinolone to 7-hydroxyquinolone was tested. At 10 mg/kg dexamethasone increased the rate of oxidative debenzoylation of the model substrate 6.4-fold, from 0.021 ± 0.004 to 0.134 0 ± 0.010 μmol/min/mg microsomal protein (mean ± SD, $n = 3$). This result suggests that the increase in *cyp3a* gene expression elicited by dexamethasone in the rat liver predicts elevated CYP3A enzyme activity. To additionally support the role of CYP3A induction in the dexamethasone-mediated hepatoprotection, another experiment was conducted in which the metabolic removal of yondelis by hepatic microsomes was investigated *in vitro*. Whereas microsomes from control rats removed 46% of yondelis substrate (1 μM) within 20 min, microsomes from dexamethasone-pretreated rats metabolized 92% of the substrate under these conditions. Therefore, it is conceivable that dexamethasone-mediated induction of CYP3A contributes to the protection by the glucocorticoid against the hepatotoxicity of yondelis in rats.

Effect of Pretreatment with Dexamethasone on Levels of Yondelis in Liver and Plasma. The experiments described above hint at the possibility that dexamethasone-induced CYP3A enzymes in rat liver oxidize yondelis to a nonhepatotoxic species at a faster rate than livers of rats that have not been pretreated with dexamethasone. If this hypothesis was correct, one may expect that increased metabolism of yondelis after pretreatment with dexamethasone at hepatoprotective doses leads to decreased yondelis levels in the liver compared with levels observed after yondelis alone. Levels of yondelis were measured in liver and plasma of rats that had been pretreated with an optimally hepatoprotective dose of dexamethasone (10 mg/kg), and these levels were compared with those in animals that received yondelis alone. Fig. 5 shows that levels of yondelis in livers of animals pretreated with dexamethasone were dramatically lower than those observed in livers from animals that did not receive the corticosteroid. Whereas liver levels in rats 1 h after administration of yondelis alone were as high as 37 pmol/g of tissue, such levels in dexamethasone-pretreated animals did never exceed 5 pmol/g of tissue. So dexamethasone pretreatment reduced hepatic yondelis levels to 14% of those in unpretreated rats 1 h after dosing. The mean values

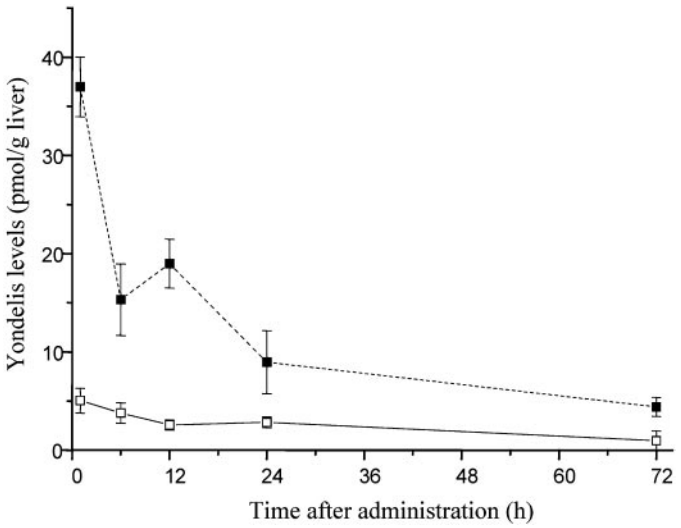

Fig. 5. Effect of pretreatment with dexamethasone on the disposition of yondelis in liver tissue of female Wistar rats. Values reflect yondelis levels in the liver of rats that received yondelis (40 μg/kg, i.v.) 24 h after either dexamethasone (10 mg/kg p.o.; □) or glycerol formal, the vehicle for dexamethasone (■). Values are the mean of four rats; *bars*, ±SD. For details of treatment and HPLC-mass spectrometry analysis see "Materials and Methods."

for the AUC_{liver} (between 0 and 72 h after dosing) were 501 and 108 pmol/g × h ($n = 4$) for animals that received yondelis without pretreatment or after dexamethasone, respectively. In contrast, dexamethasone pretreatment neither changed the shape of the yondelis plasma concentration *versus* time curve (data not shown) nor decreased systemic exposure to yondelis, as adjudged by the AUC_{plasma} (between 0 and 6 h after dosing); mean AUC values were 2.2 and 2.3 pmol/ml × h ($n = 4$) in animals that received yondelis alone or after dexamethasone pretreatment, respectively. In a confirmatory experiment the effect on yondelis plasma disposition of a higher dose of dexamethasone (50 mg/kg) given on 3 consecutive days preceding yondelis was investigated. Under these conditions plasma AUC values of yondelis in unpretreated and dexamethasone-pretreated rats were 1.3 and 2.2 pmol/ml × h ($n = 4$), respectively. Thus, yondelis levels in dexamethasone-pretreated rats were not lower than those obtained in animals on yondelis alone.

DISCUSSION

The results presented above demonstrate unambiguously that yondelis-induced liver damage is amenable to pharmacological abrogation, at least in the rat. Pretreatment of rats with high-dose dexamethasone afforded dramatic protection against the detrimental effect of yondelis as reflected by plasma levels of bilirubin and of liver enzymes AST and ALT, and by histopathogical changes. Dexamethasone also abolished almost all of the alterations in hepatic gene expression that accompanied yondelis-mediated liver damage. Most importantly, dexamethasone did not interfere with the antitumor activity of yondelis, as in none of the five tumor models studied was the antitumor activity of the combination inferior to that of yondelis alone. Interestingly, in three tumor models the combination was clearly more efficacious than yondelis alone. The timing of administration of dexamethasone in relation to that of yondelis was shown to be crucial, as coadministration failed to protect the rat liver against yondelis-induced changes, and may even have exacerbated them somewhat.

Dexamethasone possesses pleiotropic pharmacological activities including activation of many transcription factors and anti-inflammatory stimuli, many of which could conceivably contribute to its ability to protect rat livers from the adverse effect of yondelis. The findings presented above permit a preliminary insight into the potential mechanism by which dexamethasone protects the rat liver against yondelis. Firstly, pretreatment with the corticosteroid engendered a dramatic suppression of hepatic yondelis levels and, thus, effectively decreased hepatic exposure to the drug. Secondly, this suppression may have been the corollary of the propensity of dexamethasone to induce CYP3A enzymes and, thus, to increase the metabolic removal of yondelis. Consistent with a hepatoprotective role of CYP3A induction are results obtained after pretreatment of rats with phenobarbital or indole-3-carbinol, the former an inducer of several CYPs including CYP3A, the latter a specific CYP3A inducer. Whereas phenobarbital did not protect livers from the deleterious effect of yondelis, indole-3-carbinol (0.1 or 0.5% in the diet) was, like dexamethasone, hepatoprotective.[5] Yondelis is a substrate of CYP3A4 *in vitro* (23, 24), although rigorous data to support the relevance of this enzyme in the disposition and/or antitumor activity of yondelis *in vivo* is still lacking. Human cell microsomes that express CYP3A4 have been shown to metabolize yondelis to three species, *N*-desmethylyondelis (ET-729) and two molecules generated by oxidative degradation of the drug (24). It is conceivable that the accelerated generation of some of these species, or of other nontoxic metabolites, is responsible for the hepatic depletion of yondelis in rats that have been pretreated with dexamethasone. In our study yondelis metabolites were not investigated; therefore, we cannot discard alternative explanations, such as that dexamethasone exerts its effect, at least in part, via increased bile flow and consequent elevated rate of yondelis secretion. Nevertheless it is important to stress that dexamethasone pretreatment failed to decrease yondelis plasma levels, compared with those observed after administration of yondelis alone. This finding suggests that the systemic availability of yondelis is not confounded by high-dose dexamethasone, which, in turn, is consistent with the uncompromised antitumor activity of the combination compared with that of yondelis alone. Furthermore, the findings taken together suggest that, whereas dexamethasone pretreatment decreases hepatic levels of yondelis effectively, it does not interfere markedly with the step(s) that determine(s) the rate at which yondelis is eliminated from the systemic circulation. It is conceivable that yondelis levels in the liver represent such a relatively small fraction of the total dose of yondelis in the body, that an increase in hepatic clearance caused by dexamethasone failed to affect its plasma AUC markedly.

It needs to be emphasized that the results described here have been obtained in the female rat, the animal model with the highest sensitivity toward yondelis-induced liver changes. Manifestations of damage induced by yondelis in human liver are less severe than those reported in the female rat (7), and hepatic changes are generally reversible in patients. Therefore, it is conceivable that details of the mechanism by which yondelis induces hepatic damage differ between rats and humans, and that such differences in turn engender discrepant susceptibilities toward amelioration by dexamethasone. For that reason the conclusions drawn from the results obtained in rats should be extrapolated to the clinic only with extreme prudence. High-dose dexamethasone therapy is feasible, as it is used clinically in conditions in which immunosuppression and anti-inflammation are desired, such as systemic lupus erythematosus, renal transplantation, steroid-resistant nephrotic syndrome, and crescentic glomerulonephritis. Long-term use of dexamethasone can lead to diabetes mellitus, especially in older patients, and other adverse effects of high-dose dexamethasone include hypertension, arrhythmias, hypokalemia, psychosis, and sus-

[5] Donald, Verschoyle, Greaves, Orr, Jimeno, Gescher, unpublished observations.

ceptibility to infection, but these effects are often manageable (25, 26).

In conclusion, the results of the experiments described above emphatically support the notion that pretreatment with a single high dose of dexamethasone prevents the deleterious effects of yondelis on function, structure, and gene expression in the rat liver without compromising its experimental antitumor activity. The timing of the pretreatment seems to be crucial for optimal protective potency, with a 24-h pretreatment interval being apparently most propitious, and simultaneous dosing probably enhancing toxicity. These results provide a powerful rationale to evaluate carefully the potential clinical usefulness of pretreatment with high-dose dexamethasone in cancer patients who receive yondelis, with the aim to suppress its unwanted hepatic effects.

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Complete Protection by High-Dose Dexamethasone against the Hepatotoxicity of the Novel Antitumor Drug Yondelis (ET-743) in the Rat

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