Title
Antiprogestins and cytochromes P450 3A

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Publication Date
1997

Peer reviewed|Thesis/dissertation

ANTIPROGESTINS AND CYTOCHROMES P450 3A: METABOLISM, INACTIVATION AND REGULATION STUDIES

by

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DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

PHARMACEUTICAL CHEMISTRY

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA

San Francisco

[redacted]

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Degree Conferred: . . . . . .

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ACKNOWLEDGMENTS

I would like to convey my utmost appreciation to my advisor, Dr. Leslie Z. Benet, for his tireless and enthusiastic leadership and guidance. I feel very privileged, indeed, to have benefited and learned from his tremendous energy, positive outlook, unflagging support and considerable wisdom. I could not have chosen a better mentor.

I would also like to thank Drs. Deanna Kroetz and Betty-ann Hoener for their very timely and insightful reading of this dissertation - their suggestions improved it notably. Thank you as well to Drs. Almira Correia, Kathleen Giacomini, Frank Szoka and Martin Shetlar for advice and for serving on my orals committee (along with Dr. Kroetz), making the experience, surprisingly, relatively enjoyable. Thanks also to Dr. Svein Øie and former members of his laboratory (Drs. Peter Swaan and Kate Hillgren, and Magnhild Lange) for their instructive collaboration on the cell culture studies, and to Dr. Bob Upton for his confidence in my ability to help instruct the departmental PK course, an experience that has benefited me greatly.

I owe a great deal to numerous members (past and present) of the Benet lab and the Department for their friendship, encouragement and help over the years: Drs. Robert Z. Harris and Parnian Zia-Amirhosseini, Dr. Rae Yuan, Laurent Salphati, Dr. Mark Grillo, Dr. Vikram Ramanathan, Tina Glisovic and, of course, the incomparable Ms. Vivian Tucker.

Heartfelt thanks also go to my parents, Raymond and Patricia, and to my sisters and brother, Liz, Carolyn, and Stuart, for their love and support. Merci à mes beaux-parents, Michel et Danièle, et à mon beau-frère Eric, pour m'avoir accueillir si chaleureusement dans la famille.

And finally, above all, I would like to thank my wonderful wife Marie, for her total and unconditional love, support, encouragement and friendship, without whom I wouldn't have made it through the last 4 1/2 years.

ABSTRACT

Antiprogestins and Cytochromes P450 3A:
Metabolism, Inactivation and Regulation Studies

Graham Richard Jang

Antiprogestins are a relatively new class of therapeutic agents with tremendous promise as antineoplasts and in fertility regulation. Their numerous potential uses warrant a thorough understanding of their metabolism and the effects they have on the activity and expression of their metabolizing enzyme(s). The *in vitro* metabolism of mifepristone (RU 486, the first developed antiprogestin) in microsomes from 3 human liver donors reflected metabolite profiles observed *in vivo*, with predominance of the mono-N-demethylated metabolite and lower levels of didemethylated and hydroxylated derivatives. The initial rates of formation of these metabolites were reduced by up to 82% by chemical inhibitors selective for Cytochrome P450 (CYP) 3A enzymes and by polyclonal antibodies raised to CYP3A4. These rates also correlated significantly with relative immunodetectable levels of CYP3A and with rates of CYP3A marker substrate metabolism in a bank of microsomes from 14 liver donors. The newer antiprogestins, lilopristone and onapristone, were metabolized in the presence of human liver microsomes by N-demethylation, which was ~70-80% inhibited by chemical inhibitors and antibodies specific to CYP3A enzymes. The 3 antiprogestins were found to inactivate CYP3A4 in a time and NADPH-dependent manner, consistent with their oxidation to reactive nitroso species which complex the heme of the enzyme. Potential induction of CYP3A4 by the antiprogestins was assessed utilizing transient transfections of a plasmid construct, containing 1.2kb of the *CYP3A4* 5'-flanking region and a luciferase reporter gene, into the human hepatoma cell lines HepG2 and Huh-7. Upon treatment with known CYP3A4 inducers (e.g., dexamethasone), these cell lines proved incapable of supporting *CYP3A4* transcriptional activation. Cotransfection of an expression vector for the glucocorticoid receptor was without effect, whereas further addition of a vector for the receptor DNA-binding domain surprisingly resulted in 4-6 fold

induction by dexamethasone, a modest but significant response to mifepristone (which is also a potent antiglucocorticoid) and no response to lilopristone or onapristone. These results suggest that mifepristone may induce CYP3A4 through a similar mechanism to dexamethasone and are consistent with lack of induction by the newer compounds due to their diminished interactions with the glucocorticoid receptor. Collectively, the findings of this work indicate potential drug-drug interactions and time-dependent non-linearities in antiprogestin pharmacokinetics due to CYP3A4 mediated metabolism of and inactivation by these agents, and also suggest that induction of the enzyme may further distinguish mifepristone from the newer compounds.

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Chapter 1 ANTIPROGESTINS

1.1 'Discovery' of Mifepristone

Even prior to the complete characterization of the steroid hormones and their cognate receptors in the 1960s, it was recognized that antagonists of the actions of these molecules could have a profound impact on human health and reproduction [1]. Of the 5 classes of steroid hormones: glucocorticoids, progestins, estrogens, androgens and mineralocorticoids, antagonists for the latter three were indeed discovered before the molecular basis of hormone action (receptors, DNA binding, transcriptional activation, etc.) had been fully elucidated. With the realization that a progesterone antagonist could significantly enhance human fertility regulation, the search for an antiprogestin was ongoing for nearly 20 years, initiated by the attempts of Pincus in the early 1960s [2]. Similarly, a glucocorticoid antagonist was also lacking and actively pursued.

In that context, the eventual discovery of the potent antiprogestational properties of mifepristone (Fig. 1.1, more commonly known as RU 486, a shortened form of the company numeric designation RU38486) was a somewhat serendipitous end to two lengthy and nontrivial pursuits. This compound was initially synthesized in 1980 by chemists at Roussel-Uclaf under a research effort to develop antiglucocorticoids [3]. Among a series of other 11β-substituted 19-norsteroids, it was singled out for further development due to its greatest relative affinity to the glucocorticoid receptor (GR). Screening of the compound for binding to the other classes of steroid receptors led to the fortuitous discovery that it also bound avidly to the progesterone receptor (PR). These early studies demonstrated relative binding affinities roughly 5 and 3-fold greater than progesterone and dexamethasone (a potent synthetic glucocorticoid) to the rabbit uterine PR and rat thymus GR, respectively [4]. Mifepristone also binds the rat prostate androgen receptor (AR) with approximately one-fourth the affinity of testosterone and can thus also act as a weak antiandrogen; it does not bind appreciably to either the estrogen (ER) or mineralocorticoid receptor (MR) (relative binding affinities < 0.1% compared to the

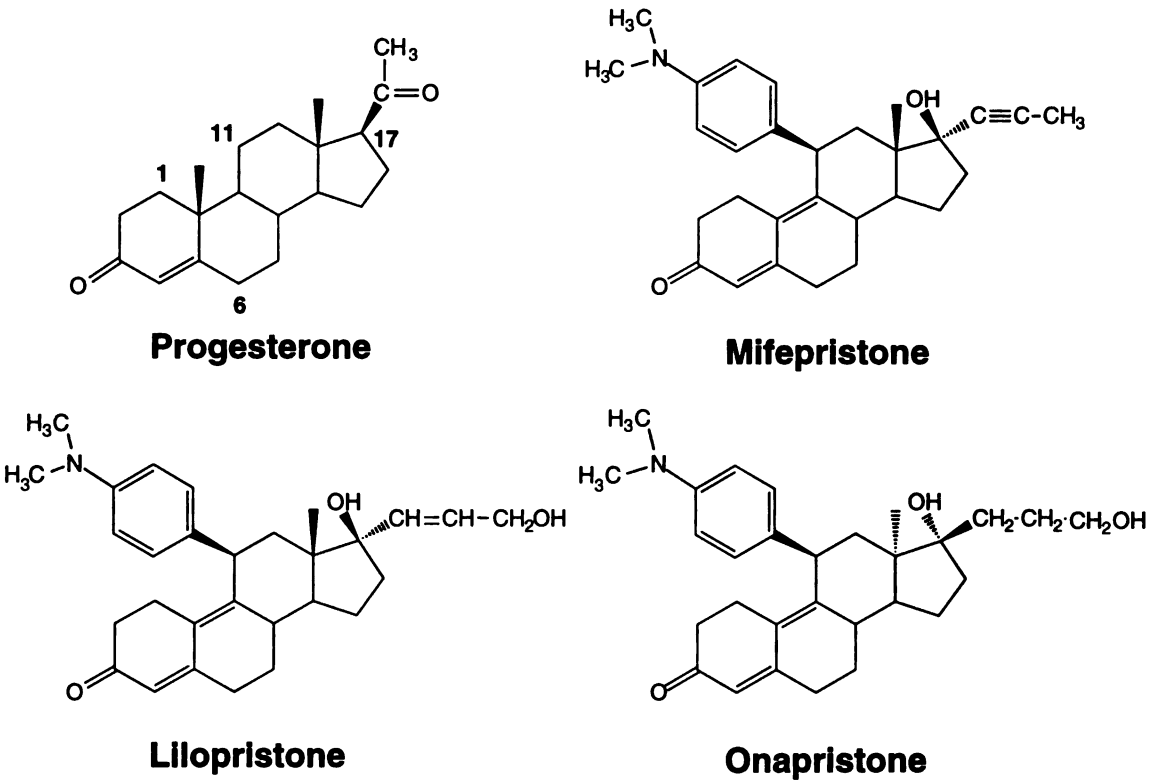


Figure 1.1 - Structures of progesterone and the three antiprogestins mifepristone (RU 486), lilopristone and onapristone. The 11β-dimethylaminophenyl substituent of the antihormones is thought to be crucial for their antagonist activity.

endogenous ligands) [4]. The observation that this antihormone binds both PR and GR is not surprising given that progesterone also binds GR and can act as a weak antiglucocorticoid.

1.2 Development as an Abortifacient

From its initial discovery, it was logical to envision the eventual use of mifepristone in the medical termination of pregnancy. This application as a "contragestive" agent had first been proposed for an antiprogestin in the early 1960s [1], based on the crucial role of progesterone in both the initiation and maintenance of pregnancy. Clearly, the initial required steps were the demonstration of antagonist activity *in vivo* and successful termination of pregnancy in experimental species. The former was demonstrated in the immature female rabbit, wherein progesterone induced changes in endometrial morphology (proliferative to secretory phase) were dose-dependently and completely antagonized by orally administered mifepristone [4]. Pregnancy termination was first demonstrated in the rat and other small animal models and subsequently in a non-human primate (macaque monkey) [5].

Shortly thereafter, the first clinical trial was carried out in 1981 with 11 women at less than 49 days gestation receiving 600 mg of the drug orally [6]. There was complete expulsion of the embryo in 9 of the women treated. Subsequently, larger trials confirmed that 200-600 mg mifepristone administered orally at less than 49 days amenorrhea was efficacious in roughly 50-86% of those treated [7-10], a frequency insufficient for eventual clinical use. It was then hypothesized that subsequent administration of a prostaglandin analog could increase efficacy by stimulating uterine contractions, causing expulsion of the conceptus [11]. With this dual-treatment regimen, efficacy increased to 95%. It is now known that antiprogestins sensitize the myometrium to exogenous prostaglandins and also

inhibit their degradation, leading to stronger contractrile effects on the uterus [12, 13]. Larger trials in the mid 1980s therefore utilized 400-600 mg mifepristone followed 36-48 hr later by either sulprostone (a PGE_2 analog injected intramuscularly) or gemeprost (a PGE_1 analog administered via vaginal suppository) [14, 15]. Reported efficacies were now consistently 95-100% with little untoward effects, the most notable being prolonged uterine bleeding (average duration 9 days, heavy in 4-5% of subjects but requiring transfusion in only ~0.1%) and abdominal cramping (caused primarily by the prostaglandin). This combination regimen was approved in France in September 1988 and is now also used in Great Britain, Sweden and China. It should be noted that there were reportedly 3 cases of myocardial infarction (1 fatal) among 60,000 patients in France, attributed to *i.m.* injection of sulprostone [16]. Therefore in women greater than 35 years of age, who smoke>10 cigarettes per day or who otherwise have potential cardiovascular disease the mifepristone/sulprostone regimen may be contraindicated (*i.m.* sulprostone was subsequently withdrawn from the French market).

Other recent clinical trials have demonstrated 94-95% efficacies when used at up to 63 days amenorrhea and/or with the orally administered prostaglandin E_1 analog misoprostol [17-22]. In addition to the advantage of oral formulation, misoprostol has been safely and widely used for the prevention of gastric ulcer. A review of the safety and efficacy of mifepristone when used in this capacity, as well as the myriad potential uses for antiprogestins (briefly reviewed below), was conducted by a committee organized by the Institute of Medicine [23]. Largely due to the extensive, safe and effective use of the drug in the aforementioned countries, it moved quickly into a multicenter phase III clinical trial in this country. The Food and Drug Administration recently (September, 1996) granted conditional approval of mifepristone-prostaglandin for the medical termination of pregnancy.

1.3 Newer Antiprogestational Agents

Since the initial report of the synthesis and characterization of mifepristone over 400 antiprogestins have been synthesized, most notably by Roussel-Uclaf, Schering AG and Organon. Because of the potent antiglucocorticoid properties of mifepristone, a major goal of these syntheses has been the development of agents that are selective for PR and would thus be free of potential toxicities resulting from perturbation of the hypothalamic-pituitary-adrenal axis. Thus far, all developed antiprogestins have at least some degree of antiglucocorticoid activity. However, the two Schering compounds, lilopristone and onapristone (Fig. 1.1), possess much lower activity relative to mifepristone as measured by reversal of dexamethasone induced tyrosine aminotransferase activity in rat hepatoma cells [24]. Table 1.1 summarizes this and other characteristics that distinguish these newer agents from mifepristone. Lilopristone and onapristone are also the only antiprogestins, other than mifepristone, that have been used in clinical trials [25, 26]. We thus chose to study these compounds as well because they represent potentially more selective antiprogestin therapy and are the furthest characterized and developed of the newer agents.

TABLE 1.1

Distinguishing characteristics of mifepristone, lilopristone and onapristone

	Mifepristone	Lilopristone	Onapristone
Relative binding affinity to rabbit uterine PR (promegestone =100%)	68	72	19
Reversal of dexamethasone induced tyrosine aminotransferase activity in rat hepatoma cells (%)	100	4	5
Binds α_1-acid glycoprotein?	yes	yes	no
Liganded-dimerized receptor complexes bind DNA?	yes	yes	no

1.4 Antiprogestin Mechanisms of Action

1.4.1 At the molecular level

Steroid hormone and related receptors comprise a large superfamily of ligand activated transcription factors that also include receptors for thyroid hormone, vitamin D, retinoic acid, 9-cis-retinoic acid and ecdysone. A large number of related genes have been cloned and their corresponding proteins termed "orphan receptors" because their ligands and functions remain unidentified. The exact mechanisms of transcriptional activation by this family of receptors is the subject of continued, intense research. This area has been the subject of several recent reviews [27-30] - the following only briefly summarizes currently accepted mechanisms.

Steroid hormone receptors reside in the cytoplasm or nucleus (those that are cytoplasmic become nuclear upon ligand binding) of specific target cell types. Their respective ligands enter cells through simple or facilitated diffusion. Binding to the receptors initiates a series of events that include dissociation of heat shock proteins (which act as inhibitors of receptor activation by blocking dimerization functions, inducing a non-active conformation and/or blocking the DNA-binding domain), hyperphosphorylation of the receptors, homo- or heterodimerization and chromatin (DNA) binding of the complexes at hormone response elements (or negative hormone response elements when the hormone action leads to lowered rates of transcription). These specific sequences, composed of palindromic repeats of consensus half-site sequences (TGTTCT for GR, PR, AR and MR), are located most commonly in the regulatory (upstream or 5'-flanking) region of hormone responsive genes. Binding of the hormone-receptor dimers to these regions allows coordination of other transcription factors necessary for transcriptional activation of the concerned gene (i.e., stabilization of a pre-initiation complex) - thereby bringing about the specific cellular and tissue effects of that hormone.

It should be noted that in humans PR exists in two forms, hPR-A and hPR-B. Products of the same gene but arising from different transcripts, the A form lacks 164 N-terminal amino acids (importantly, not part of the DNA or ligand-binding domains) [31]. Both forms activate transcription but have been shown to differ in relative activity in *in vitro* reporter gene systems [32]. One study has reported partial agonist effects of mifepristone when bound to hPRB but not hPRA [33]. Therefore, tissue specific expression of receptor forms could potentially alter efficacy of the compound and/or be a factor in tissue-specific effects.

Antagonism of progesterone and glucocorticoid action arises through competition with agonist for the receptor ligand-binding domain and subsequent lack of transcriptional activation. However, due to the complexity of hormone and receptor action, the effects of antiprogestins on receptor conformation and DNA binding are not simple and indeed seem to differ among antagonists. Limited proteolysis of progesterone and mifepristone bound receptors yields different fragments [34]. A monoclonal antibody that recognizes the 14 C-terminal amino acids of the ligand-binding domain is capable of binding agonist but not antagonist-bound receptors [35]. A C-terminal truncated receptor mutant is capable of binding mifepristone but not agonist [36]. Collectively, these results suggest that mifepristone binds at a distinct but overlapping site in the receptor ligand-binding domain.

Once mifepristone is bound to PR, there is dissociation of heat shock proteins, followed by dimerization and DNA-binding [37]. One study has demonstrated that mifepristone-receptor dimer complexes compete with agonist-occupied complexes for the same DNA binding site *in vivo* [33]. However, from studies with agonist or mifepristone-bound complexes bound to ^{32}P-labelled progesterone response elements (PREs), with subsequent gel electrophoresis and autoradiography, it seems that the conformations of the DNA-bound complexes differ (antagonist-bound DNA has greater mobility) [33, 37].

An early report of mifepristone treatment of HeLa cells (a human epithelial cell line) transiently transfected with hPR-A or hPR-B and a MMTV-CAT reporter plasmid (a mouse

mammary tumor virus (MMTV) promoter and chloramphenicol acetyl-transferase reporter gene; the MMTV promoter contains multiple PRE/GREs) indicated that the drug displayed no agonist activity upon formation of DNA-bound complexes [33]. However, with a simpler PRE/GRE-tk (thymidine kinase) promoter, the hPR-B (but not A) form was transcriptionally active when bound to mifepristone [33, 38]. Additionally, the C-terminal PR deletion mutant noted above that binds mifepristone but not agonist was indeed found to also activate transcription [36]. In another cell type, mifepristone displayed agonist activity only in the presence of 8-Br cAMP (which stimulates protein kinase A), implicating a role for cellular phosphorylation pathways in modulating response to antiprogestin-receptor complexes [39]. Collectively, it appears that mifepristone can display partial agonist activity depending on the receptor form present, the promoter region of the gene of interest and the cell (or tissue) type.

As noted in Table 1.1, onapristone differs with mifepristone and lilopristone in that the onapristone-receptor complexes either do not bind DNA [39, 40] or do so with 10-fold lower affinity than the mifepristone complexes [41]. In *in vitro* (cell culture) studies, under conditions in which mifepristone displays agonist activity, onapristone does not [40], consistent with an apparent lack of DNA binding. Furthermore, hyperphosphorylation of PR that occurs upon agonist or mifepristone binding does not occur with onapristone [42, 43]. Therefore, despite exhibiting lower relative binding affinity to PR than mifepristone or lilopristone, onapristone may possess similar or greater potency due to the absence of partial agonist activity. Early studies at Schering AG revealed that onapristone was more potent than mifepristone in terminating pregnancy in the rat [44] and also appeared to display greater synergistic activity with subsequently administered prostaglandins [45, 46].

1.4.2 At the organ level: in termination of pregnancy

Following ovulation, the dominant follicle in the ovary (from which the ovum was released) is transformed into the corpus luteum, which secretes progesterone during the hence-named luteal phase of the menstrual cycle. Progesterone induces transformation of the endometrium from a proliferative to a secretory state (i.e., one receptive to a fertilized ovum). Should this occur, the implanted and developing trophoblast secretes β-hCG, which acts to maintain the luteal body and therefore continued secretion of progesterone until the placenta assumes this role near the 8th week of pregnancy. Progesterone also inhibits uterine production of prostaglandins and desensitizes the myometrium to these and other contractile agents (oxytocin and vasopressin), thereby maintaining uterine quiescence.

The abortive effects of antiprogestins in early pregnancy are therefore derived from action at several levels. Firstly, antagonism of progesterone's effects on endometrial tissue leads to endometrial erosion (as occurs with menstruation). This in turn leads to detachment of the developing conceptus from the uterine wall and a decline in β-hCG secretion, which results in deterioration of the corpus luteum. With cessation of progesterone production from the corpus luteum, further endometrial deterioration occurs, accompanied by increased myometrial contractility and cervical softening/dilatation. As noted above, mifepristone also directly sensitizes the myometrium to prostaglandins. These events thus culminate in expulsion of the embryo, occurring more frequently or consistently with subsequent prostaglandin administration.

1.5 Potential Uses of Antiprogestins

Antiprogestins represent a new and tremendously promising class of therapeutic agents with a multitude of potential uses well beyond their role in the medical termination of pregnancy.

1.5.1 Endometriosis

Endometriosis is ectopic growth of endometrial tissue, most often in the pelvic cavity. The condition is non-malignant but is progressive, very painful and currently difficult to treat. An early study in which 6 subjects participated (receiving 100 mg mifepristone per day orally for 3 months) resulted in significant lessening of pelvic pain in all 6 but no evidence of disease regression by laparoscopy [47]. A lower dose (50 mg/day) administered for 6 months to 9 subjects again led to reduced pelvic pain in all subjects; however, laparoscopy revealed a (mean) decrease of 55% in American Fertility Society scores (measures of the extent of ectopic growth) in 8 of the 9 subjects [48].

1.5.2 Uterine leiomyoma (fibroids)

These tumors of the myometrium occur in roughly 20% of women over the age of 30 and are the principal indication for hysterectomy [49]. Like endometriosis, they are dependent upon ovarian hormone secretion and are thus also treatable with GnRH agonists (which down-regulate ovarian estrogen secretion). However, this induced state of hypoestrogenism leads to hot flashes and bone loss. A study with mifepristone has shown that 50 mg/day for 3 months can decrease tumor volume by a mean of 49% in 10 subjects [47, 50]. A dose of 25 mg/day (N=17) resulted in reduction of tumor volume by 68% at 12 weeks, with no evidence of unwanted glucocorticoid antagonism (i.e., no change in urinary cortisol). A dose of 5 mg/day was less effective (29.2% reduction in tumor

volume). Further studies are needed, but medical treatment of fibroids may present an alternative to hysterectomy for women who desire continued child-bearing potential.

1.5.3 Meningioma

These tumors of the meninges (membranes surrounding the brain or spinal canal) are non-malignant and slowly progressive, but can lead to neurological injury and sometimes death if not surgically removed. They are more frequent in women; their growth accelerates during pregnancy and the majority express PR (intriguingly, 81% are PR+ in women vs. only 19% in men) [51]. There is therefore evidence that progesterone may play an important role in the development and/or growth of meningioma, which presents a clear rationale for the use of antiprogestins in their treatment. Mifepristone and onapristone demonstrate cytostatic and/or cytocidal effects on meningioma cells in culture [52]. Additionally, when meningioma cells are implanted into nude mice, the 2 compounds cause tumor regression [52]. In the largest clinical trial reported, 28 patients with unresectable tumors received 200 mg mifepristone daily for up to 62 months [53]. Eight patients demonstrated a reduction in tumor volume as evidenced by computerized tomography or MRI scanning. Antiprogestins may also be helpful in cases of recurrent tumors or as an adjunct or alternative to radiation therapy.

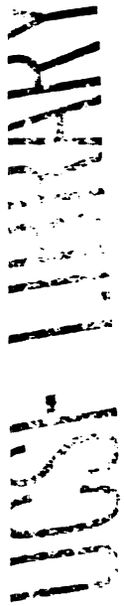
1.5.4 Breast cancer

It is now generally recognized that both estrogens and progestins have proliferative effects on normal and cancerous breast tissue. Tamoxifen, an antiestrogen, is a mainstay in endocrine therapy for ER and PR expressing, hormone-dependent tumors. Therefore, antiprogestins in theory represent new and promising agents for the treatment of breast cancer that is unresponsive to tamoxifen or other therapies.

In PR-positive breast cancer cell lines such as MCF-7 and T47D, studies have in general demonstrated dose-dependent antiproliferative and even growth-repressive effects

of mifepristone [54-56]. However, the compound has also been reported to be growth stimulatory in a certain subclone of T47D [57]. Responses of these and other cell lines varies widely among laboratories due to heterogeneity and genetic instability within the cell lines (emergence of differentially responding subpopulations with potentially mutated receptors) and varied culturing/experimental conditions (optimal vs. suboptimal growth conditions). All results in such cell lines are therefore difficult to interpret.

In animal models of breast cancer the results are somewhat clearer. Induction of mammary tumors by dimethylbenzanthracene (DMBA) in rats is delayed by co-administration of mifepristone [55]. In animals with established tumors the drug is cytostatic [58], although tumor regression required co-administration of tamoxifen [59]. Another study with DMBA-induced tumors indicated that onapristone treatment alone could produce tumor regression similar to ovariectomy and to that found with the combined mifepristone/tamoxifen treatment [60]. These effects could potentially be related to differences in receptor conformation and DNA-binding for this antagonist as noted above.



The results of only 3 clinical trials have been reported and the results are overwhelmingly unimpressive, perhaps indicating that antiprogestins might be most efficacious in combination with an antiestrogen. In the earliest report [61], 22 post-menopausal (or oophorectomized) women with metastatic breast cancer that was unresponsive to tamoxifen and radiation (or other chemo-) therapy were given 200 mg mifepristone per day. After four to six weeks treatment, stabilization or partial regression was observed in 12 subjects (53%). However, these effects were maintained at 3 months in only 4 patients (18%). Interestingly, in accord with a proposed PR-mediated mechanism, among the responders and non-responders whose tumors were evaluated for PR expression, 4/4 and 0/4 were positive, respectively. Presence of PR thus appears potentially predictive of response to antiprogestins.

A second trial involved 11 post-menopausal patients with metastatic disease treated for 3-34 weeks with 200-400 mg mifepristone daily [62]. One subject demonstrated an

objective (lymph node) response lasting 5 months, 6 patients had short-term (3-8 month) stabilization of disease and in the remaining 4 subjects the disease progressed. Finally, in the most recent study [63], 28 PR positive patients who had received no prior therapy were given 200 mg of the drug per day. A partial response (stabilization) was observed in only 3 (10.7%). The results of these trials are consistent with the animal studies wherein mifepristone alone promoted, at best, tumor stasis but not significant regression.

In summary, due to the complexity of hormone/antihormone mechanisms of action (potential agonist effects), heterogeneity in steroid receptor expression in tumors, the relative dearth of reliable *in vitro* and animal models for evaluating tumor response to these agents and the small number of clinical trials reported, the precise role of antiprogestins in breast cancer treatment is currently unclear. Future studies should address whether onapristone or combined antiprogestin/antiestrogen therapy would be more efficacious *in vivo*. Other potential uses for antiprogestins that warrant investigation are in prophylaxis (or chemoprevention) or in adjuvant therapy (after tumor resection to prevent recurrence).

1.5.5 Prostate cancer

As noted above, mifepristone also binds AR and it was thus hypothesized that the compound could have a role in the treatment of prostate cancer. Currently only two studies have been reported in which prostate carcinoma cell lines and xenografted tumors were utilized. In the first, mifepristone was found to have no effects on the growth of an androgen-sensitive cell line, LCNaP [64]. However, another study reported significant growth inhibition in LCNaP, as well as in two androgen-insensitive lines (PC-3 and DU145) [65]. Furthermore, when PC-3 cells were grafted into nude mice, complete tumor growth suppression was observed with administration of 4 mg mifepristone per 100g body weight. The compound thus appears to have some activity against prostatic carcinoma, although further studies are clearly needed to elucidate its mechanism of action and the relative activities of other antiprogestins with different binding affinities to AR.

1.5.6 Cushing's Syndrome

Due to the potent antiglucocorticoid activity of mifepristone, the drug has been evaluated for use in the treatment of hypercortisolism. Normally, the pituitary secretes adrenocorticotropic hormone (ACTH) to modulate (stimulate) cortisol production from the adrenal cortex. Increasing levels of cortisol trigger a decrease in ACTH secretion from the pituitary and therefore provide negative feedback regulation. With certain cases of ectopic (metastase) pituitary tumors that secrete ACTH, mifepristone has been demonstrated to reverse the Cushingoid phenotype (depression, hypertension) [66]. However, in pituitary-dependent disease (Cushing's disease), wherein ACTH secretion is low in response to a given cortisol level (higher setpoint), mifepristone has been demonstrated to actually cause an *increase* in ACTH and cortisol levels [67]. This is thought to occur because the drug antagonizes the cortisol-mediated negative feedback regulation at the pituitary. Use of mifepristone and newer antiglucocorticoids for non-pituitary dependent disease therefore shows promise, but requires further clinical trials.

1.5.7 Contraceptive Uses

The use of antiprogestins in fertility regulation can conceivably comprise 3 different stratagem: daily or weekly administration (to prevent ovulation and/or development of a secretory endometrium), cyclical administration (at a stage of the menstrual cycle to delay endometrial maturation or to induce menstruation) or as a post-coital agent (to prevent implantation).

Daily/weekly administration:

Studies have investigated the effects of mifepristone administered at doses of 1,2,5 or 10 mg per day for 1 month [68, 69]. Doses of 2 mg or more uniformly suppressed ovulation, whereas the 1 mg dose caused suppression in only 1 of 5 [69]. However, endometrial development was impaired in all subjects, suggesting that implantation of a

theoretical conceptus would perhaps have been compromised. In the aforementioned trial in endometriotic patients [47], ovulation was uniformly suppressed for 3 months by 100 mg mifepristone per day - indicating that the effect can be extended for longer durations. In one report, onapristone was shown to inhibit follicular development and ovulation at 15 and 50 mg/day, but did so inconsistently at 5 mg/day [25].

Mifepristone (10 or 50 mg) administered once a week for 5 weeks failed to uniformly inhibit ovulation [70]. However, other studies have demonstrated that 2 or 5 mg per week, while permitting ovulation, perturb endometrial development [71]. These and other reports demonstrate that the endometrium appears more sensitive than the pituitary-ovarian axis to the effects of antiprogestins. Continuous or intermittent administration of antiprogestins might therefore be most successful as a contraceptive method by inhibiting endometrial (but not follicular) development.

Timed administration:

A single 200 mg dose of mifepristone given early in the luteal phase (2 days after the LH surge) resulted in only 1 pregnancy among 169 cycles studied, presumably by disrupting endometrial development [72]. This method therefore appears promising but relies on self-detection of urinary LH. Theoretically, antiprogestins could also be given at the end of the luteal phase to induce menstruation. However, studies with mifepristone when used in this capacity revealed unacceptable failure rates of 3-16% [73, 74].

Post-coital administration:

Two large clinical trials have been performed to evaluate the efficacy of 600 mg mifepristone administered within 72 hours of unprotected intercourse (regardless of menstrual cycle stage) [75, 76]. Both of these compared mifepristone to the currently available Yuzpe regimen (high dose estrogen [ethinyl estradiol] and progestin [levonorgestrel]). Compiling the results of the two studies [77] reveals that there were no

pregnancies among the 597 patients receiving mifepristone, whereas 9 of 589 on the Yuzpe regimen conceived. Importantly, fewer patients in the mifepristone group reported nausea (37-40% vs. 60-70%), vomiting (3% vs. 17-22%) or breast tenderness (18-27% vs. 18-46%). However a greater number of those receiving mifepristone experienced a delay in the subsequent cycle, most likely because the large dose and long $t_{1/2}$ of the drug caused delay in follicular development once therapy had been discontinued. A lower dose may be similarly efficacious but avoid this undesirable effect. The use of antiprogestins as post-coital agents thus appears superior to currently available methods.

Other possible indications for antiprogestins, not discussed here, are in instances of ectopic pregnancy or intrauterine fetal death (to induce abortion), for cervical ripening/dilatation (to facilitate uterine access) and induction of labor at term.

1.6 Mifepristone Pharmacokinetics in Humans

The pharmacokinetics of mifepristone in man exhibit dose and time dependent nonlinearities, partly influenced by saturable plasma protein binding. The drug is administered orally and has variable bioavailability of 30-56% [78]. Various studies report rapid absorption, with maximal concentrations (C_{max}) attained in 0.7-2.2 hours [79, 80]. With doses lower than 100 mg, C_{max} and area under the concentration-time curve (AUC) increase proportionately [80]. For doses of 100-800 mg, however, no changes in C_{max} (~2-4 μM) are observed, while concentrations of the three major metabolites (mono-, didemethylated and hydroxylated derivatives) do increase dose-dependently [80]. Parent drug AUC increases non-proportionately for doses greater than 100 mg, and once C_{max} is attained the concentrations obey zero-order kinetics for 24-48 hours. The investigators hypothesized that doses greater than 50 mg (which do not exhibit zero-order kinetics) result

in concentrations that saturate α_1-acid glycoprotein (AAG) binding sites, leading to a higher fraction unbound (f_u) and greater tissue extravasation of the drug [80]. Because metabolite levels (most notably the mono-N-demethylated derivative) peak very early, usually exceed those of parent drug and display dose-proportionality, it would appear that intestinal and first-pass metabolism are very significant and are not saturated at these doses.

Receptor binding studies have revealed that 94-99% of mifepristone is bound to AAG at sub-saturating levels (< ~2.5 μM) [79]. Partly due to this low f_u, steady state volume of distibution and clearance estimates in man are low (10% body weight and 0.55 L/kg/day, respectively) relative to rat (135% body weight and 72 L/kg/day) [78], a species in which the drug does not bind AAG. Mifepristone does not bind cortisol or sex-steroid binding globulins [81], but has been shown *in vitro* to bind albumin, albeit weakly [82]. The elimination $t_{1/2}$ of mifepristone (following the zero-order elimination phase) in humans is highly variable, with several studies reporting values of 24-54 hours [78, 83-85].

As noted above, mifepristone administered alone is relatively ineffective at terminating pregnancy (~14-50% failure rates). One study found no differences in plasma concentrations of parent drug, the 3 major metabolites or AAG between responders (N=13) and non-responders (N=4) [86]. Therefore non-response to mifepristone is likely not due to differences in pharmacokinetics or metabolism in the intestine or liver.

1.7 Mifepristone Metabolism - Studies in the Rat

Mifepristone is metabolized in the rat primarily via sequential demethylations of the 11β-dimethylaminophenyl group and hydroxylation of the 17α-propynyl moiety (Fig. 1.2) [78]. These three derivatives are also the major metabolites in man. Relative binding affinities (mifepristone=100%) of the mono-,didemethylated and hydroxylated metabolites to rat uterine PR were 75, 8 and 9%, respectively; the corresponding relative binding

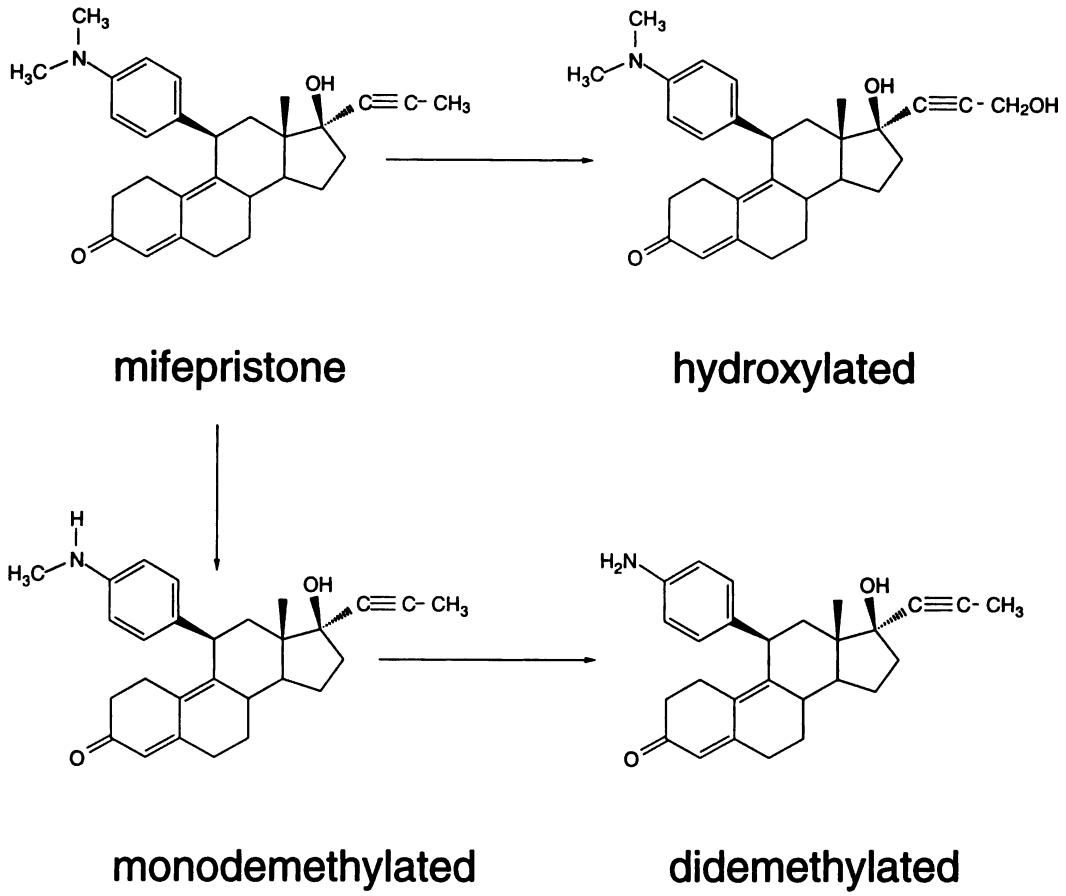


Figure 1.2 - Major routes of metabolism for mifepristone in rat and man, via successive demethylations of the 11β-dimethylaminophenyl and hydroxylation of the 17α-propynyl groups.

affinities to rat thymus GR were 98, 39 and 42% [78]. Affinities of these metabolites to human uterine PR were 21, 9 and 15% and to human placental GR 61, 45 and 48%, respectively [79]. These *in vitro* binding studies therefore indicate that the metabolites have lower affinity to human PR than progesterone (itself 43% relative to mifepristone) but higher affinity to GR than cortisol (9% relative to mifepristone) - suggesting that the pool of metabolites might collectively play a role in the *in vivo* antiglucocorticoid but not antiprogestin action of administered mifepristone.

At the initiation of this work, the enzyme(s) involved in the formation of these principal metabolites had been investigated only in the rat. The involvement of cytochromes P450 (CYPs) was demonstrated using carbon monoxide and aminoglutethimide as non-selective CYP inhibitors [87]. Microsomes from rats treated with inducers of CYPs1A (methylcholanthrene), CYPs 2B, 2C and 3A (phenobarbital) and CYPs 2C and 3A (pregnenolone) degraded mifepristone at rates 16, 161 and 166%, respectively, of those observed in microsomes from control rats, indicating that enzymes of the 2B, 2C and 3A subfamilies were likely involved. However, antibodies raised to phenobarbital-induced, purified CYP2B1 completely inhibited mifepristone metabolism in a subsequent work [88], suggesting that in the rat CYP2B1 plays a major role in the metabolism of the antiprogestin. The related human isoform CYP2B6 comprises only ~0.2% of expressed liver CYPs [89] and its role in xenobiotic metabolism appears very limited [90]. Further work with rat hepatoma variants that expressed only 2B and 2C enzymes [91] or that reportedly expressed 3A forms [92] led these investigators to conclude that CYPs 2B and 2C primarily demethylate whereas CYP3A hydroxylates the compound. Given that CYP mediated steroid oxidations in the rat are known to involve multiple subfamilies [93-95], these results are difficult to interpret and should not be extrapolated to man. Oxidation of steroids at alternating positions around the A-D rings is diagnostic of different rat CYP subfamily activities and does not apply to human forms.

1.8 Cytochrome P450 3A4

Despite the implications of multiple CYP subfamily involvement in mifepristone metabolism in the rat, we hypothesize that CYP3A4 is likely the principal enzyme catalyzing the oxidations of mifepristone, lilopristone and onapristone in human liver.

A study utilizing 11 recombinant human CYPs (discussed in more detail in the following chapter) revealed that CYP3A enzymes catalyzed testosterone, progesterone and androstenedione hydroxylations with the highest rates relative to enzymes of other subfamilies [96]. This finding suggests that it is possible that most synthetic steroidal and antisteroidal molecules are also CYP3A4 substrates. Other data consistent with, but by no means indicative of, CYP3A4 mediated metabolism of mifepristone are its relatively low and variable oral bioavailability (noted above), which may be partly due to CYP3A4 mediated metabolism of the drug in the intestine and liver (and the relatively high variability at which it is expressed in those tissues).

A principal role of CYP3A4 in antiprogestin metabolism could have very important clinical implications. The enzyme is involved in the metabolism of greater than 50% of all xenobiotics metabolized by the CYPs in humans. Its active site accommodates very structurally diverse drugs such as nifedipine [97], the immunosuppressants cyclosporine [98] and tacrolimus [99], midazolam and triazolam [100], the antiarrhythmic agents lidocaine [101], amiodarone [102, 103] and quinidine [104], taxol [105], etoposide [106], vinblastine and other vinca alkaloids [107, 108]. Therefore, due to the promising potential of mifepristone and the newer antiprogestins and the resulting likelihood of their chronic or long-term administration, CYP3A4 involvement in their metabolism implies potentially significant drug-drug interactions.

We further hypothesize that the three antiprogestins are capable of inactivating CYP3A4, principally due to the presence of a tertiary amine as the principal site of their metabolism and the propensity of CYP3A4 to form reactive nitroso metabolites from these

functional groups (this hypothesis is further discussed in the introduction to Chapter 4). Moreover, we believe that mifepristone, but not lilopristone or onapristone, may induce CYP3A4 via its interactions with the glucocorticoid receptor (discussed in Chapter 5). Induction of the protein could also have important clinical implications for the long-term use of the drug, and further distinguish the newer agents from mifepristone.

1.9 Rationale and Specific Aims

Antiprogestins are a relatively new and promising class of therapeutic agents. Most of their potential uses (as discussed above) would entail their long-term or chronic administration. We thus thought it important to characterize their metabolism *in vitro* utilizing human liver microsomes, to identify the principal enzyme(s) involved in their metabolism and to assess what effects they have on the activity and expression of the active enzyme(s). In addition to mifepristone, we chose to study lilopristone and onapristone because of their reduced antiglucocorticoid activity and more advanced characterization relative to the other newer agents. We hypothesize that CYP3A4 is the principal enzyme catalyzing antiprogestin oxidations in humans and that, furthermore, these compounds are capable of inactivating the enzyme. Additionally, we believe that mifepristone has the potential to modulate CYP3A4 expression. The specific aims of this thesis were therefore:

(1) To characterize the *in vitro* metabolism of mifepristone, lilopristone and onapristone in human liver microsomes and compare this with metabolite formation observed *in vivo*.

(2) To identify which CYP(s) are principally involved in the three antiprogestins' oxidations in human liver microsomes, hypothesizing that it is CYP3A4.

(3) To assess potential enzyme inactivation by these agents via formation of a reactive nitroso species (through demethylation of the 11β-dimethylaminophenyl moiety).

(4) To evaluate the suitability of the human hepatoma cell lines HepG2 and Huh-7 for the mechanistic study of CYP3A4 regulation by hormones and antihormones.

Chapter 2 CHARACTERIZATION OF MIFEPRISTONE METABOLISM IN HUMAN LIVER MICROSOMES

2.1 Summary

The metabolism of mifepristone in human liver microsomes was characterized and evidence obtained supporting cytochrome P450 (CYP) 3A4 as the principal enzyme catalyzing its oxidation. Human liver microsomes from 3 donors catalyzed the demethylation of mifepristone with mean (± S.D.) apparent K_m and V_{max} of 10.6 ± 3.8 μM and 4920 ± 1340 pmol/min/mg protein, respectively; the corresponding values for hydroxylation of the compound were 9.9 ± 3.5 μM and 610 ± 260 pmol/min/mg protein. A variety of complementary approaches were then used to elucidate the major CYP catalyzing these reactions: chemical and immunoinhibition of specific CYPs; correlation analyses between initial rates of mifepristone metabolism and relative immunodetectable CYP levels and rates of CYP marker substrate metabolism; and evaluation of metabolism by cDNA-expressed CYP3A4. Progesterone and midazolam (CYP3A4 substrates) inhibited metabolite formation by up to 77%. The CYP3A inhibitors gestodene, triacetyloleandomycin and 17α-ethynylestradiol inhibited mifepristone demethylation and hydroxylation by 70-80%; antibodies to CYP3A4 inhibited these reactions by approximately 82 and 65%, respectively. In a bank of human liver microsomes from 14 donors, rates of mifepristone metabolism correlated significantly with relative immunodetectable CYP3A levels, rates of midazolam 1'- and 4-hydroxylation and rates of erythromycin N-demethylation, markers of CYP3A catalytic activity (all $r^2 \geq 0.85$ and $P<0.001$). No significant correlations were observed for analyses with relative immunoreactive levels or marker catalytic activities of CYP1A2, CYP2C9, CYP2C19, CYP2D6 or CYP2E1. Recombinant CYP3A4 catalyzed mifepristone demethylation and hydroxylation with apparent K_m values 7.4 and 4.1 μM, respectively. Collectively, these data demonstrate *in vitro* metabolite formation reflective of that observed *in vivo* and clearly support CYP3A4 as the enzyme primarily responsible for mifepristone demethylations and hydroxylation.

2.2 Introduction

Cytochromes P450 (CYPs) comprise a large superfamily of hemoproteins found in both pro- and eukaryotes [90]. In man, they are expressed in most tissues and catalyze the oxidation of endogenous substrates (such as cholesterol, steroids and fatty acids), environmental carcinogens (polycyclic aromatic hydrocarbons, nitrosamines) and many drugs. Among the reactions catalyzed by CYPs are dehalogenation, N-hydroxylation, S-, N-, and O-dealkylations and aliphatic and aromatic hydroxylations.

As noted above (Chapter 1), mifepristone is metabolized via N-demethylations and hydroxylation in both experimental species and man. It was therefore highly likely that these reactions are catalyzed by CYPs in the liver, and the aforementioned work in the rat indeed confirmed general CYP involvement [87]. In the rat liver, numerous CYPs (including members of the 2A,2B,2C,2D and 3A subfamilies) vigorously catalyze regiospecific steroid hydroxylations [93-95]. However, among 11 cDNA-expressed human CYPs, CYP3A enzymes possessed the overwhelmingly highest steroid hydroxylase activities towards testosterone, progesterone and androstenedione [96]. The steroids were hydroxylated primarily at the 6β position, although oxidations at the 2β and 15β (for testosterone) and 16α (of progesterone) positions also occurred. CYPs 2C8 and 4B1 (the latter expressed only in the lung) demonstrated activities roughly 1/20 and 1/3 those of CYP3A4 for these three steroids, whereas CYP1A2 was found to 2β-hydroxylate estradiol at a similar rate. Because members of the CYP3A subfamily are therefore considered the major CYPs involved in endogenous steroid metabolism, we hypothesized that CYP3A4 in the liver would assume this role for mifepristone, and that the implication of rat CYP2B and 2C enzyme involvement should not be extrapolated to man.

CYP3A4 is involved in the metabolism of greater than 50% of all xenobiotics metabolized by the CYPs in humans. The enzyme accommodates very structurally diverse drugs including nifedipine [97], the immunosuppressants cyclosporine [98] and tacrolimus

[99], midazolam and triazolam [100], the antiarrhythmic agents lidocaine [101], amiodarone [102, 103] and quinidine [104], taxol [105], etoposide [106], vinblastine and other vinca alkaloids [107, 108]. Therefore, coupled with the promising potential of mifepristone and the resulting likelihood of its chronic or long-term administration, CYP3A4 involvement in its metabolism could have important implications for potential drug-drug interactions.

Because studies with recombinant enzymes alone can be misleading due to inherent overexpression of CYPs present in the liver in very low amounts and the consequent absence of potentially more active forms, we chose to carry out these studies in human liver microsomes utilizing the following approaches: chemical and immunoinhibition of specific isoforms; correlation analyses between rates of mifepristone metabolite formation and relative immunodetectable CYP levels and rates of CYP isoform marker substrate metabolism; and evaluation of metabolism by cDNA-expressed CYP3A4.

2.3 Materials and Methods

2.3.1 Chemicals & specimens

Mifepristone and its monodemethylated and hydroxylated metabolites were a gift from Roussel Uclaf (Romainville, France). Didemethylated mifepristone and gestodene were kindly supplied by Schering AG (Berlin, Germany). Midazolam was a gift from Hoffmann-LaRoche (Nutley, NJ). 7,8-Benzoflavone, quinidine, sulfinpyrazone, 17α-ethinyl estradiol, troleandomycin, disulfiram, progesterone, deoxycorticosterone, NADPH and sodium phosphate were purchased from Sigma Chemical Co. (St. Louis, MO). Furafylline was obtained from Research Biochemicals International (Natick, MA). HPLC grade methanol and acetonitrile were from Fisher Scientific (Pittsburgh, PA).

Human liver specimens were obtained from organ donors, all of whom had died as a result of head trauma, under a protocol approved by the Committee on Human Research of the University of California at San Francisco. Microsomes were prepared by homogenization and differential centrifugation, following established methods [109], of non-transplantable liver from a 53 year old male (HL-01), 5 year old male (HL-02) and 36 year old female (HL-03). The microsomes were stored until use at -80°C in 10 mM Tris acetate (pH 7.4) containing 1 mM EDTA and 20% (w/v) glycerol. Protein and CYP concentrations were determined by Pierce bicinchoninic assay (Pierce Chemical Co., Rockford, IL) and Fe^{2+} vs. Fe^{2+}-CO difference spectra [110], respectively.

The bank of human liver microsomes from 14 donors used for correlation analyses (designated HL-A through N) and rabbit polyclonal antibodies used in immunoinhibition experiments were kind gifts of Dr. Steven A. Wrighton (Eli Lilly & Company). This bank of microsomes has been previously described and characterized for relative immunoreactive CYP levels and initial rates of CYP isoform marker substrate metabolism [111-113]. Microsomes from a human β-lymphoblastoid cell line stably transfected to coexpress CYP3A4 and NADPH-CYP reductase were obtained from Gentest Corp. (Woburn, MA).

2.3.2 Assay for mifepristone and metabolites

A published HPLC assay for the determination of mifepristone and its three major metabolites in serum [86] was modified for measuring levels in microsomal incubations. Briefly, the mobile phase was methanol : acetonitrile : water (35:30:35) at a flow rate of 1.4 ml/min through a Beckman Ultrasphere C-18 column (5 μm x 4.6 mm i.d. x 250 mm) with UV monitoring (304 nm). The autoinjector, pump and detector were Shimadzu models SIL-9A, LC-600 and SPD-6A, respectively. A Hewlett Packard 3392A integrator was used. Quantitation was done with extinction coefficients from authentic standards.

2.3.3 Incubation conditions

In general, incubations consisted of 60 μg microsomal protein (or 200 μg protein for microsomes containing cDNA-expressed CYP3A4) in 0.1 M Na_2HPO_4 buffer (pH 7.4) at 37°C with substrate (mifepristone or its monodemethylated metabolite in the absence or presence of inhibitors) added in methanol (final concentration $\leq$ 2%, v/v). Reactions were initiated by adding NADPH in buffer (1 mM final concentration, total reaction volume 200 μl) after a 5 min pre-incubation period, stopped after 2 min by adding a 2-fold volume of acetonitrile containing deoxycorticosterone as internal standard and vortexed. Precipitated proteins were pelleted by centrifugation (5 min at 11,000g) and 100-150 μl of the supernatant subjected to HPLC.

For mechanism-based inhibitors, catalysis dependent inactivation was initiated by addition of NADPH (using HL-03 microsomes) and carried out for 30 min, followed by ten-fold dilution of the microsomes with buffer containing mifepristone and NADPH. Thereafter, reactions were stopped at 2 min and samples processed as described above. In some experiments, inhibition of the second demethylation was evaluated using the monodemethylated metabolite (synthetic standard) as substrate.

In immunoinhibition experiments, various amounts of sera from pre-immune and immunized rabbits (to CYP2C9 and CYP3A4) were incubated with HL-02 microsomes at

24°C for 30 min before the addition of substrate and the assay of catalytic activity. The antisera to CYP2C9 was found to be maximally inhibitory (by approximately 75%) of tolbutamide hydroxylation at 75 µl/mg protein (S.A. Wrighton, personal communication).

2.3.4 Data analysis

For characterization of metabolite formation, substrate concentration was varied up to 200 µM and kinetic parameters estimated by non-linear regression analyses (with Minim 1.8a) assuming single enzyme Michaelis-Menten kinetics with a weighting factor equal to the reciprocal of the observed initial rate. No evidence of biphasic kinetics was observed in Eadie-Hofstee plots. Correlation analyses were performed by linear regression using a commercially available statistics program (Statworks 1.2). All results are presented as the means of duplicate determinations ± half the range.

2.4 RESULTS

2.4.1 HPLC assay and preliminary incubations

Modification of the reported assay for measuring plasma levels of mifepristone and its major metabolites resulted in good peak shape and separation. Figure 2.1 shows (A) a representative chromatogram from injection of mifepristone, synthetic standards of the three metabolites and deoxycorticosterone (the internal standard) and (B) a chromatogram from an injection following a 2-min incubation of microsomes from HL-02 in the presence of 100μM mifepristone at 37°C. Sample processing was as described in Materials and Methods.

The initial incubation conditions were developed with HL-02. As expected from metabolite formation patterns observed *in vivo*, monodemethylated metabolite formed most quickly and extensively; levels of hydroxylated and didemethylated metabolites remained lower throughout the observed incubation periods. Product formation was linear up to approximately 0.4 mg protein/ml and 3 minutes and was not affected by substitution of an NADPH-generating system or NADPH concentrations greater than 1 mM. Thus a protein concentration of 0.3 mg/ml, an incubation period of 2 min and 1 mM NADPH were routinely used to insure initial rate conditions.

2.4.2 Kinetics of metabolite formation

Figure 2.2 depicts a representative Michaelis-Menten plot and data output from Minim 1.8a for the first demethylation of mifepristone in microsomes from HL-01. Table 1 summarizes the Michaelis-Menten parameter estimates for demethylation and hydroxylation in microsomes from this liver sample, HL-02, HL-03 and β-lymphoblastoid cells expressing CYP3A4. For the microsomes from the three human livers, the mean (± S.D.) apparent K_m and V_{max} values for the first demethylation were 10.6 ± 3.8 μM and 4920 ± 1340 pmol/min/mg protein, respectively; the corresponding values for

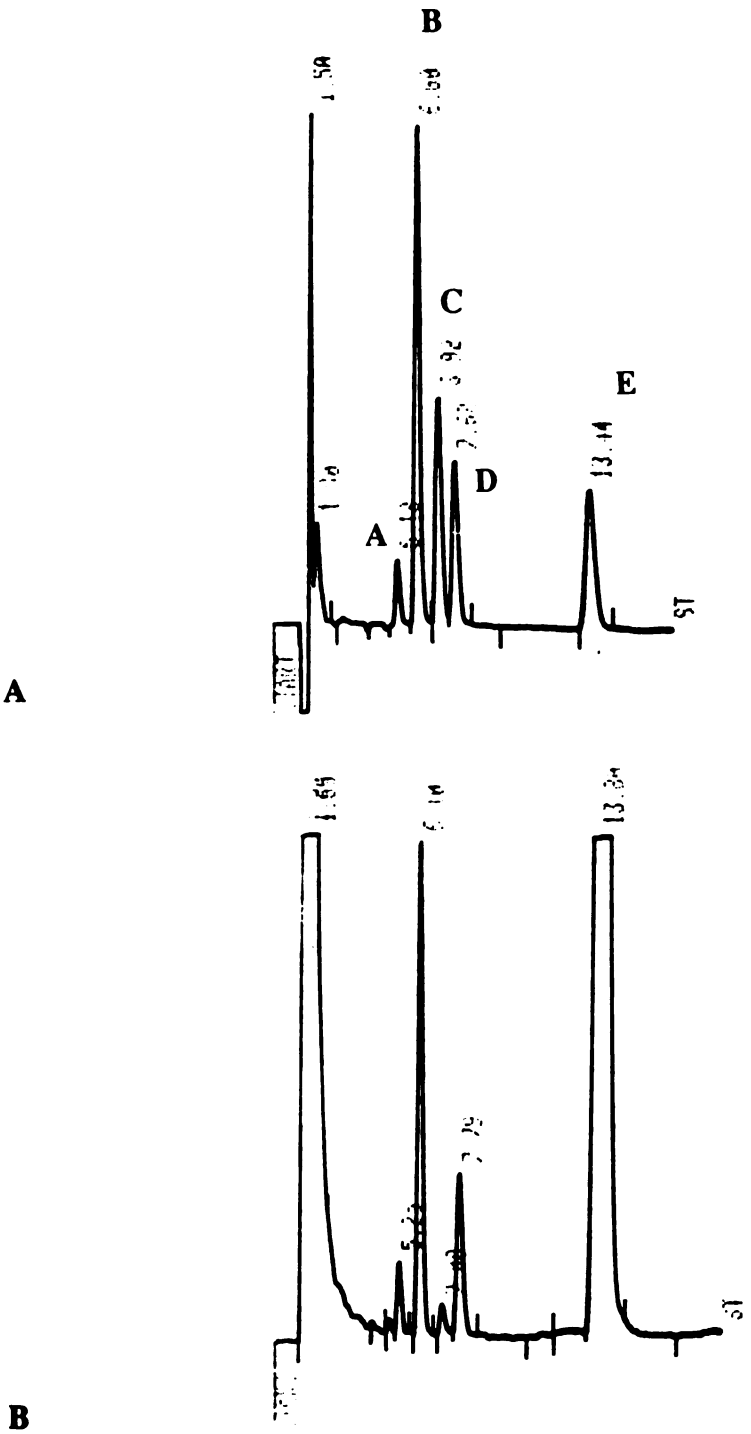


Figure 2.1 **(A)** HPLC chromatogram of mifepristone (peak E) and synthetic standards of its monodemethylated (D), hydroxylated (C) and didemethylated (A) metabolites; peak B is the internal standard deoxycorticosterone. **(B)** HPLC chromatogram of a sample following a 2 min (37°C) incubation of 100μM mifepristone with microsomes from HL-02.

Minim 1.8a 11:58:35 03-10-1995 Gauss-Newton-Marquardt
Least sum of squared errors Parameters scaled Explicit derivatives Hartley's interpolation
Data-file:- HL-01/DM/avg
Function: a*x/(b + x)
2 parameters estimated 9 data points Convergence criterion 1E- 4
Variance model (1/|y obs|^p) f = 1 p = 1
Converged after 7 iterations (5s).

Parameter	Final value	± S.D.	Initial guess
a	3372.443	152.7728	3070.1424
b	14.51501	1.723724	9.63698

Singular values of Jacobian matrix (largest/smallest = 5.75561):
5899.661 1025.028

Approx. correlation matrix:
1.0000
0.8623 1.0000

Objective = 115333.2 R-squared = .9767429
Estimated S.D. of residuals (d.f. 7) = 128.3595
A.I.C. = 108.9002

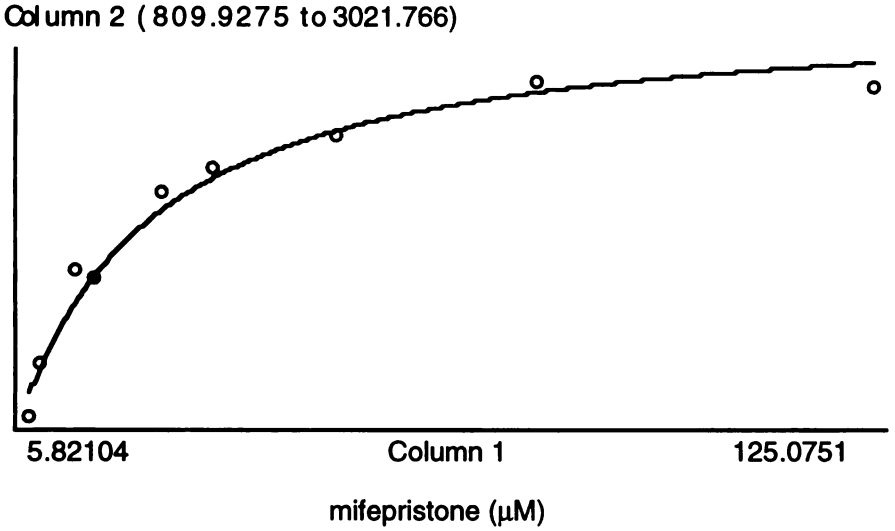


Figure 2.2 Representative Michaelis-Menten plot and data output from Minim 1.8a for the demethylation of mifepristone in microsomes from HL-01. Each data point represents the mean of duplicate determinations; the y-axis denotes the initial rate of demethylation in pmol/min/mg microsomal protein (values are not shown in a Minim plot).

Table 2.1 Estimated Michaelis-Menten parameters for mifepristone demethylation and hydroxylation in human liver microsomes and in microsomes containing recombinant CYP3A4 and NADPH-CYP reductase*.

	monodemethylation			hydroxylation			CL_{int} ratio
	K_m	V_{max}	CL_{int}	K_m	V_{max}	CL_{int}	dm/hyd
HL-01	**14.5**	**3370**	**232**	**13.3**	**310**	**23**	**10.1**
HL-02	**10.3**	**5750**	**558**	**9.9**	**800**	**81**	**6.9**
HL-03	**6.9**	**5640**	**817**	**6.4**	**720**	**112**	**7.3**
rCYP3A4	**7.4**	**1140**	**154**	**4.1**	**110**	**26**	**5.9**

*Apparent K_m, V_{max} and CL_{int} values are expressed in μM, pmol/min/mg protein and μl/min/mg protein, respectively.

hydroxylation were 9.9 ± 3.5 μM and 610 ± 260 pmol/min/mg protein. The microsomes containing cDNA-expressed CYP3A4 catalyzed the two oxidations with similar apparent K_m but lower V_{max} values (normalized to protein). A comparison of the relative *in vitro* CL_{int} (ratio of V_{max}/K_m) for the two metabolic pathways reveals a consistent 6 to 10 fold greater rate of elimination via demethylation.

2.4.3 Effects of chemical inhibitors on metabolite formation

The following competitive inhibitors were tested (with their CYP isoform specificities): sulfinpyrazone (CYP2C9), quinidine (CYP2D6), progesterone and midazolam (CYP3A4/5). Sulfinpyrazone and quinidine up to concentrations of 100 μM did not inhibit mifepristone demethylation, while progesterone and midazolam over the same concentration range did so by 77 and 66%, respectively (Fig. 2.3). 7,8-Benzoflavone (up to 100 μM) dose-dependently inhibited demethylation of the antiprogestin (Fig. 2.3). This compound is less selective for CYP1A2 than furafylline, has been reported to inhibit CYP2C9 [114] and has been found to activate or inhibit some CYP3A4 reactions [114-116]. Importantly, while the flavone more selectively and potently (by ~90%) inhibits CYP1A2 at low (<10 μM) concentrations [114], little inhibition (<18%) was observed in our studies at these concentrations (Fig. 2.3). The result is therefore more consistent with inhibition of CYP3A4 rather than CYP1A2 and was later confirmed using furafylline (see below).

The effects of the following quasi-irreversible (TAO) or mechanism-based inhibitors were evaluated: furafylline (CYP1A2), disulfiram (CYPs 2A6, 2B6 and 2E1), gestodene (CYP3A4/5), troleandomycin (CYP3A4/5) and 17α-ethinyl estradiol (CYP3A4). The compounds selective for CYP3A enzymes significantly inhibited demethylation by 70-80% (Fig. 2.4) and had nearly identical effects on hydroxylation (Fig. 2.5A). Moreover, 17α-ethinyl estradiol and troleandomycin inhibited the second demethylation to the same extent observed for the other two oxidations (Fig. 2.5B). Disulfiram and furafylline did

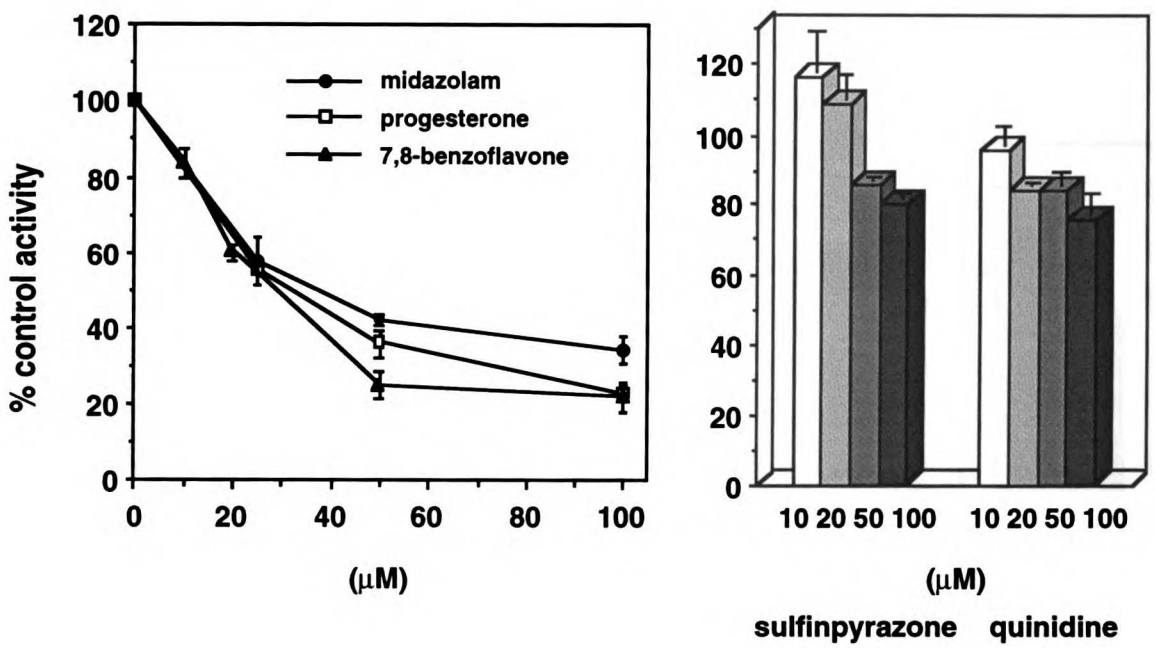


Figure 2.3 Substrates of CYP3A enzymes (midazolam and progesterone) inhibit mifepristone demethylation whereas inhibitors of CYP2C enzymes (sulfinpyrazone) and CYP2D6 (quinidine) have little effect. 7,8-Benzoflavone also inhibits the demethylation, which is likely due to nonspecific interactions with CYP3A4.

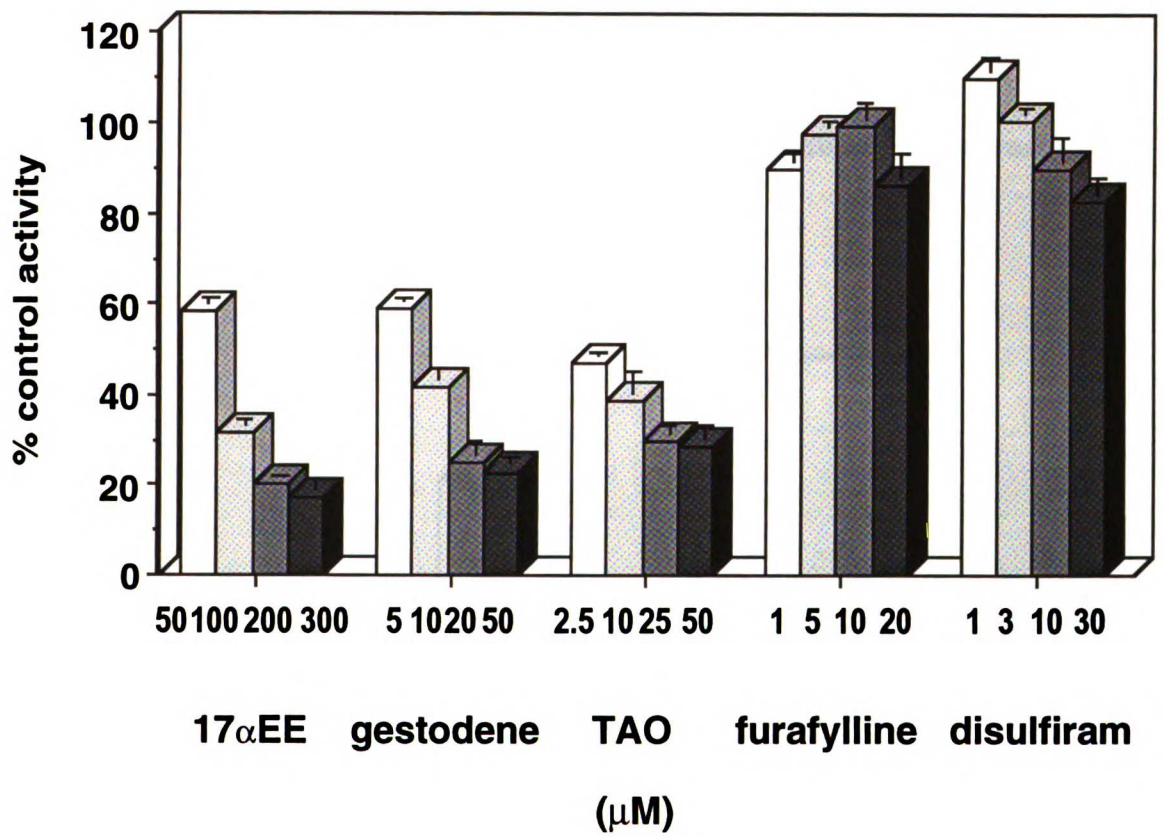
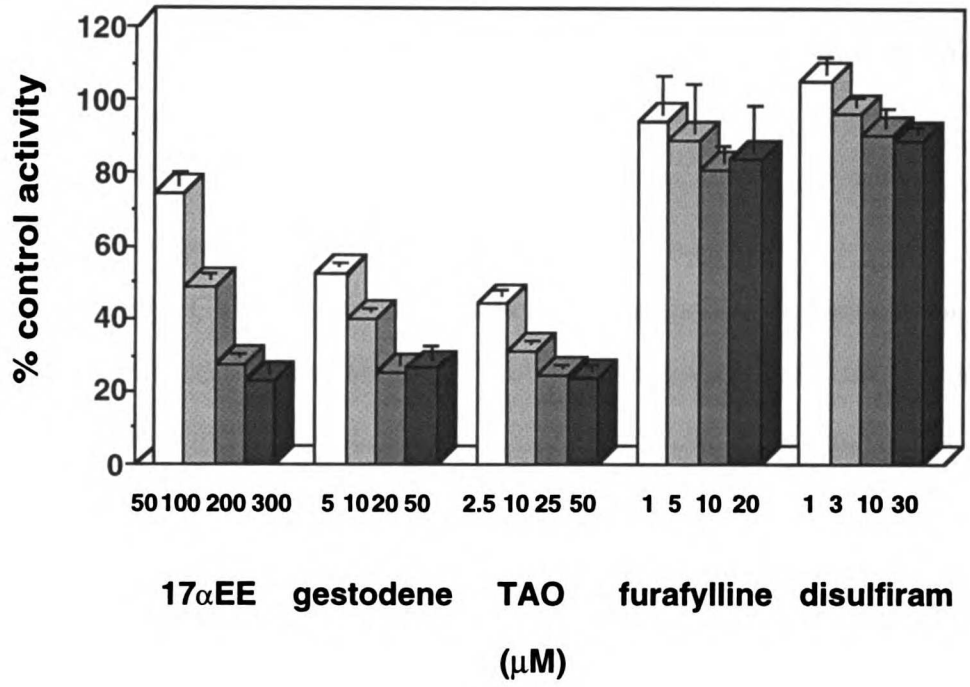


Figure 2.4 Effects of mechanism-based or quasi-irreversible (TAO, troleandomycin) CYP inhibitors on initial rates of mifepristone demethylation in microsomes from HL-03 (17αEE, 17α-ethinyl estradiol).

A



B

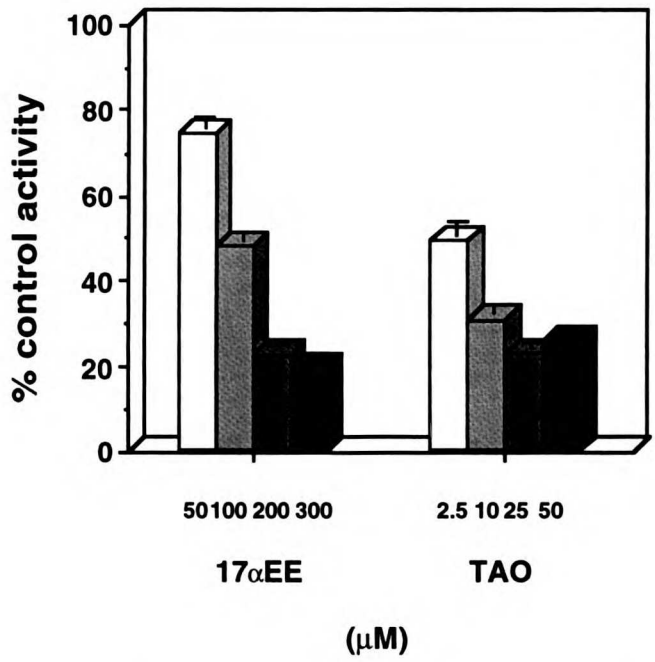


Figure 2.5 **(A)** Inhibition of mifepristone hydroxylation by inhibitors selective for CYP3A enzymes and lack of inhibition by those specific to other isoforms **(B)** 17α-Ethinyl estradiol and troleandomycin mediated inhibition of the second demethylation step using the monodemethylated derivative as substrate.

not significantly inhibit demethylation or hydroxylation (Fig. 2.4). The minor (11-17%) inhibition observed with these compounds is likely due to slight inhibition of CYP3A4 at these concentrations [114, 117].

2.4.4 Immunoinhibition experiments

Antibodies to CYP3A4 strongly inhibited both mifepristone demethylation (~82%) and hydroxylation (~65%) as shown in Fig. 2.6A. We assessed the effects of antibodies to CYP2C9 because previous work in the rat [87, 88, 91, 92] had implicated CYP2C enzymes. These antibodies, as well as pre-immune sera (PI), had no effect on either biotransformation (Fig. 2.6B).

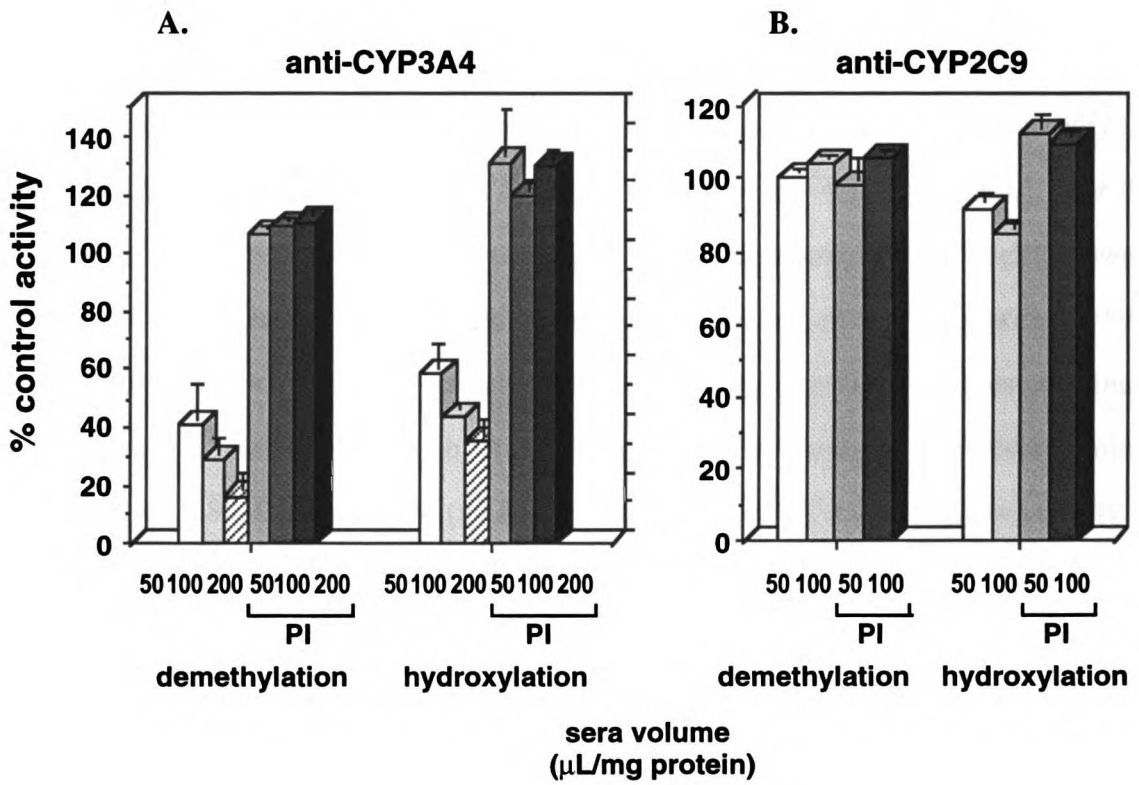


Figure 2.6 **(A)** Inhibition of mifepristone demethylation and hydroxylation by rabbit polyclonal antibodies to CYP3A4 and lack thereof by pre-immune sera (PI) in microsomes from HL-02. **(B)** Lack of inhibition of either oxidation by antibodies to CYP2C enzymes.

2.4.5 Correlation analyses with relative CYP levels and rates of marker substrate metabolism

The donor characteristics, relative immunoreactive CYP levels and relative CYP marker activities for the microsomes from HL-A to N were previously determined and published [111-113] and are listed in Tables 2.2-2.4. Initial rates of mifepristone first and second demethylations and hydroxylation in human liver microsomes HL-A through N correlate very well with relative CYP3A levels (Fig. 2.7A), with rates of midazolam 4-hydroxylation (Fig. 2.7B) and 1'-hydroxylation (Fig. 2.7C), with rates of erythromycin N-demethylation (Fig. 2.7D) and with each other (Fig. 2.8). Rates of the second demethylation were determined using the synthetic, monodemethylated derivative as the substrate. The correlation analyses with rates of midazolam hydroxylation depicted in Figs. 2.7B and C were carried out excluding the samples known to contain CYP3A5 in addition to CYP3A4 (HL-E,F,G). CYP3A5, which is polymorphically expressed in only ~20-30% of adult human livers [118, 119], is known to have marked regioselectivity for hydroxylation of midazolam at the 1'-position relative to the 4-position [120]. For the analyses with midazolam 4-hydroxylation, inclusion of these samples only slightly lowers correlation coefficients for the first and second demethylations and hydroxylation to 0.97, 0.90 and 0.93 (all $P<0.001$), respectively. Inclusion of the microsomal samples containing CYP3A5 in the analyses with 1'-hydroxylation lowers the respective coefficients more noticeably to 0.83, 0.77 and 0.76 (all $P<0.001$). This may reflect the regioselectivity of CYP3A5 for midazolam hydroxylations and an apparent lack of similar regioselectivity for oxidations of mifepristone.

No significant correlations were observed between metabolite formation rates and relative immunodetectable levels of CYPs 1A2, 2D6 and 2E1 (r^2 range 0.00-0.21, mean ± S.D. = 0.10 ± 0.08, all $P>0.05$, data not shown). Additionally, no correlations were observed between initial rates of metabolite formation and rates of ethoxyresorufin O-deethylation (CYP1A2), coumarin 7-hydroxylation (CYP2A6), S-warfarin 7-hydroxylation

General Information Regarding Liver Donors

Patient code	Gender	Age	Smoking habits (pk-yr)[a]	Medical/ drug history
A	M	25	7	None
B	M	50	35	None
C	M	22	UK	Ethanol (0.25%) (unspecified drug abuse)
D	M	31	NS	None
E	M	14	NS	Pentobarbital (coma induced 1 week before death)
				Pancuronium Br
				Dopamine
				Furosemide
				Mannitol
				Heparin
				Cefazolin
F	F	50	Heavy	Alcoholic
				Insulin
G	F	48	UK	Teldrin
H	F	28	UK	None
I	M	43	NS	Phenobarbital
				Phenytoin
				Propranolol
J	F	55	UK	None
K	M	23	UK	Ethanol (0.056%)
				Dopamine
L	F	58	UK	Dopamine
				Mannitol
				Argininevasopressin
M	M	18	UK	Ethanol (0.273%)
				Alcohol rehab
N	M	21	UK	Ethanol (0.08%)
				Dopamine
				Argininevasopressin
				Diabetic

[a]Abbreviations: pk-yr, pack-year (defined as 1 package of cigarettes per day for a year); UK, unknown; NS, nonsmoker.

Table 2.2 General liver donor characteristics and medical/drug histories for HL-A to N used in correlation analyses [111-113]. Note that donors E and I received pento- and phenobarbital, respectively, known CYP3A inducers.

Relative Levels of 8 Cytochromes P450 in a Bank of Microsomes from 14 Human Livers

Liver specimen	1A2	2A6	2C8	2C9	2D6	2E1	Total 3A	3A5
A	100	100	100	100	100	100	100	0
B	74	143	106	123	371	80	110	0
C	72	139	209	130	338	154	118	0
D	30	389	84	133	431	173	73	0
E	38	355	196	72	724	74	262	26
F	64	395	167	77	264	64	192	28
G	80	264	87	101	361	29	142	32
H	94	192	46	93	184	73	73	0
I	53	718	149	217	302	147	315	0
J	65	139	230	265	291	54	121	0
K	57	569	39	124	0	115	92	0
L	45	31	38	55	73	61	66	0
M	33	224	36	88	325	88	86	0
N	96	132	87	81	0	87	98	0
Range	3×	23×	6×	5×	10×	6×	5×	—

Table 2.3 Previously determined, relative immunoreactive CYP levels in microsomes from HL-A to N [111-113]. Microsomes from HL-E and HL-I contain the highest relative CYP3A levels.

Cytochrome P450-Mediated Catalytic Activities of a Panel of Microsomes from 14 Human Livers

Liver specimen	Ethoxyresorufin *O*-deethylase	Coumarin 7-hydroxylase	*S*-Mephenytoin 4′-hydroxylase	Bufuralol 1′-hydroxylase	*N*-Nitroso-dimethylamine *N*-demethylase	Erythromycin *N*-demethylase	Total P450
A	60.0	33	112	28.5	777	193	356
B	16.6	280	17	41.5	481	168	204
C	30.8	230	180	34.9	1043	106	303
D	17.4	740	17	75.0	1120	63	284
E	19.3	600	310	87.6	505	520	507
F	28.6	520	2	36.9	528	268	329
G	23.0	410	65	55.7	333	156	293
H	33.2	330	66	31.5	635	149	293
I	27.1	780	60	42.0	1374	960	487
J	30.8	360	260	47.2	309	218	288
K	22.4	940	81	18.7	528	205	288
L	7.6	110	<2	20.1	499	119	147
M	9.4	400	39	56.9	606	112	270
N	27.1	430	237	22.3	522	243	390
Range	8×	28×	>155×	5×	4.5×	15×	3.5×

Note. All activities are expressed as pmol of product per mg microsomal protein per minute, except total P450 content which is pmol P450 per mg of protein (assay methods referenced in text).

Table 2.4 CYP isoform marker catalytic activities of microsomes from HL-A to N [111].

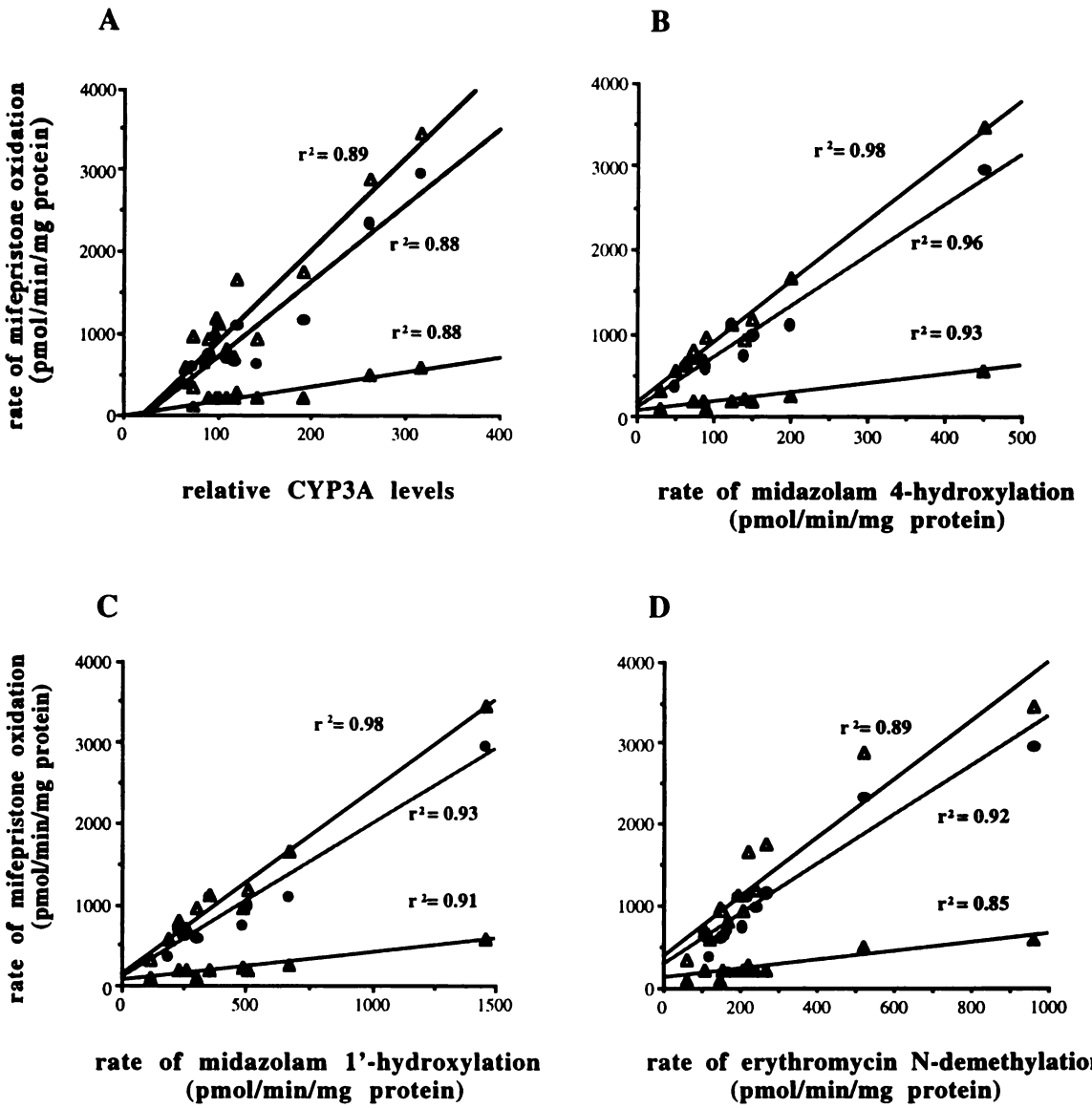


Figure 2.7 Correlations between mifepristone first (Δ) and second (l) demethylations and hydroxylation (s) and **(A)** relative immunodetectable CYP3A levels, **(B&C)** rates of midazolam 4- and 1'-hydroxylation and **(D)** rate of erythromycin N-demethylation in human liver microsomes (all $P<0.001$).

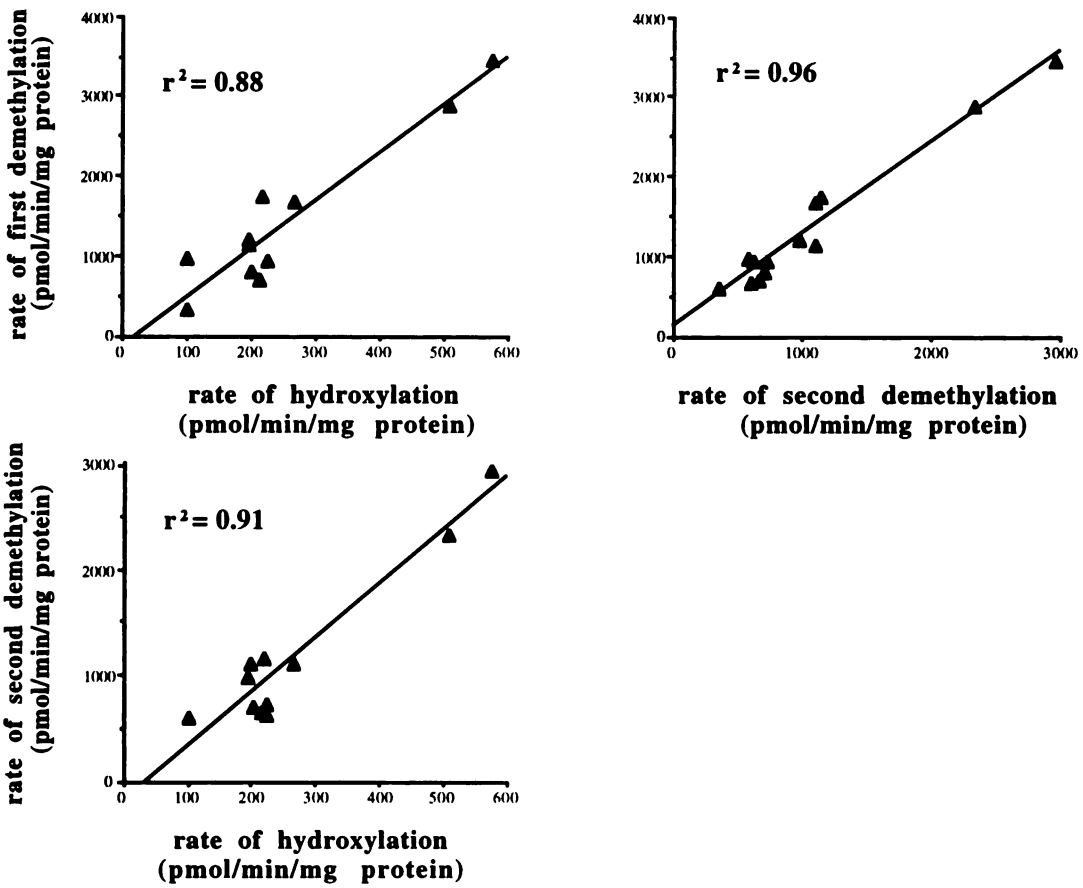


Figure 2.8 Respective correlations between initial rates of mifepristone metabolite formation in human liver microsomes (all $P<0.001$).

(CYP2C9), S-mephenytoin 4'-hydroxylation (CYP2C19), bufuralol 1'-hydroxylation (CYP2D6) and N-nitrosodimethylamine N-demethylation (CYP2E1) (r^2 range 0.00-0.28, mean ± S.D. = 0.11 ± 0.09, all $P>0.05$, data not shown).

We should note that correlation analyses of rates of first and second demethylations and hydroxylation with relative immunoreactive CYP2A6 levels determined previously [111-113] resulted in r^2 values of 0.33, 0.45 and 0.32, respectively. The correlations for the two demethylations are significant ($P<0.05$) but that for the hydroxylation is not ($P=0.06$). It is likely that these correlations stem from an inherent reciprocity between relative levels of CYP3A and CYP2A6 in this bank of human liver microsomes ($r^2=0.41$, $P<0.02$). With this in mind, any CYP3A4 catalyzed reaction in these microsomes would be expected to correlate weakly with CYP2A6 levels. Indeed, similar weak but significant correlations with CYP2A6 are also observed for initial rates of erythromycin N-demethylation ($r^2=0.46$, $P<0.01$) and midazolam 4-hydroxylation ($r^2=0.35$, $P<0.03$), both well established marker activities of CYP3A4. Furthermore, the weak correlations for mifepristone demethylations are inconsistent with the lack of correlation observed with rates of coumarin 7-hydroxylation (all $P>0.05$ as noted above) and the lack of inhibition by disulfiram at concentrations that have been shown to inhibit CYP2A6 by >70% [117].

2.5 DISCUSSION

In this work, complementary lines of evidence were obtained that collectively support CYP3A4 as the major CYP isoform catalyzing mifepristone demethylations and hydroxylation in human liver microsomes. Chemical and immunoinhibition of CYP3A4 resulted in significant inhibition of mifepristone metabolism, which was further confirmed through correlation analyses. Furthermore, a recombinant form of CYP3A4, like the metabolizing microsomal enzyme, appeared to oxidize mifepristone preferentially to the demethylated derivative (as evidenced by a higher CL_{int} relative to that of hydroxylation). Conversely, inhibition of other CYP isoforms had no effect on mifepristone metabolism, which was again consistent with the results of correlation analyses. Thus, unlike that reported in the rat [87, 88, 91, 92], enzymes of the CYP2C and CYP2B subfamilies do not appear to be involved in mifepristone metabolism in humans. This is consistent with what has been observed for the human CYPs involved in endogenous steroid metabolism (i.e., a predominant role of CYP3A4), and may thus indicate that most synthetic antihormones are likely CYP3A4 substrates as well. Indeed, tamoxifen (an antiestrogen) is metabolized primarily by CYP3A4 [121].

The weak but significant correlations observed between rates of mifepristone demethylation and relative immunoreactive CYP2A6 levels illustrate the need to supplement data from correlation analyses with that from other lines of experimentation. An inherent weakness in performing correlation analyses is the possibility of apparent but likely artifactual relationships in a particular bank of microsomes. A very small role for CYP2A6 in mifepristone metabolism cannot be completely ruled out, but is refuted by the observed lack of biphasic kinetics (at concentrations up to 50-fold those observed *in vivo*), lack of correlation with CYP2A6 marker activity and a lack of inhibition by disulfiram. Moreover, the very high levels of significance of correlations with CYP3A4 levels and activity probes, integrated with the inhibition results, argue clearly for a principal (and thus clinically

important) role of CYP3A4. This demonstrates the critical importance of evaluating the entire body of evidence to reach a conclusion about the principal CYP catalyzing the metabolism of a drug.

It was recently reported that CYP3A7, heretofore considered fetal liver specific, was detected at the protein and mRNA levels in endometrium (of pregnant and nonpregnant women) and placenta [122]. CYP3A4 and CYP3A5 were not detected in these tissues. CYP3A7 shares some substrate specificity with CYP3A4 and is known to oxidize one steroid, dehydroepiandrosterone 3-sulfate, at an apparently greater rate [90, 123, 124]. The expression of CYP3A7 in these extrahepatic tissues was variable, but seemed to increase during the menstrual cycle and with gestation length. When used as an abortifacient, mifepristone derives its effect primarily through antagonism of receptors in the endo- and myometrium. We hypothesize that instances of non-response to mifepristone when used in this capacity could be related in part to CYP3A7 mediated, target tissue metabolism of the compound. Such differences in response to mifepristone could not be attributed to differences in drug or metabolite plasma levels or levels of α_1-acid glycoprotein (to which mifepristone is highly bound) [86]. It seems plausible to also suggest that this isoform may influence the efficacy of antiprogestins when they are used as contraceptives or for endometriosis.

Given the numerous and promising potential uses of mifepristone (and other antiprogestins), the finding that CYP3A4 is its major metabolizing enzyme in human liver suggests the likelihood of drug-drug interactions subsequent to long-term administration of the compound. This is notably exemplified by the implications for its potential anticancer uses, since several current antineoplastic agents are also CYP3A4 substrates. Knowledge of this, combined with its potential for inhibiting P-glycoprotein *in vivo* (as discussed in the following chapter) could lead to more rational and effective use of this compound.

Chapter 3 CHARACTERIZATION OF LILOPRISTONE AND ONAPRISTONE METABOLISM - *CONFIRMATION OF CYP3A4 INVOLVEMENT DESPITE STRUCTURAL DIVERGENCE*

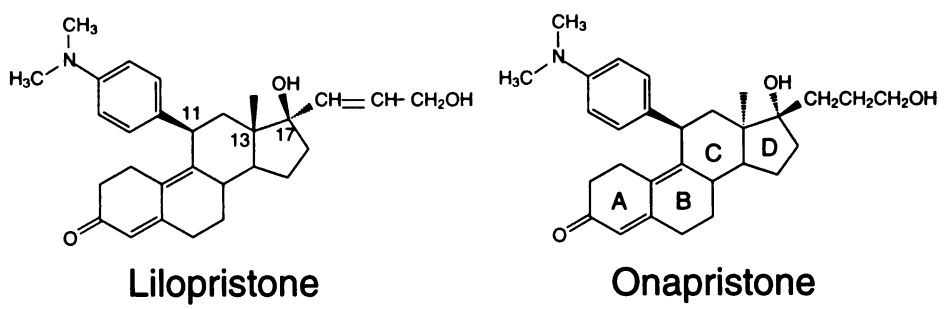
3.1 Summary

Lilopristone and onapristone are two newer antiprogestational agents that in some aspects differ significantly from mifepristone. We previously demonstrated (Chapter 2) a principal role of CYP3A4 in the oxidative metabolism of mifepristone in human liver microsomes. The goal of the present work was to assess whether the structural differences of these newer antihormones alter their CYP specificity. We hypothesized that because of the relative promiscuity of the CYP3A4 active site these newer agents would also be substrates of the enzyme despite reported alterations in their binding to other proteins. Kinetic studies with microsomes from 3 organ donors indicated lack of biphasic kinetics, consistent with a single enzyme mediating the oxidations. Selective chemical inhibitors of CYPs 1A2 (furafylline), 2C9 (sulfaphenazole), 2D6 (quinidine) and 2E1 (diethyldithiocarbamic acid) did not affect initial rates of metabolism of either steroid. Gestodene and triacetyloleandomycin (selective for CYP3A enzymes) inhibited the demethylations of both antiprogestins by up to 77%. Rabbit polyclonal antibodies to CYP3A4 decreased initial rates of N-demethylation of the antihormones by up to 82%, whereas antibodies to CYP2C9 were not inhibitory. Collectively, these data indicate that like mifepristone, lilopristone and onapristone are CYP3A4 substrates, and further suggest potential drug-drug interactions of these promising new therapeutic agents with concomitantly administered CYP3A4 substrates.

3.2 Introduction

The potent antiglucocorticoid properties of mifepristone have in some instances been manifest as unwanted effects (increase in ACTH and cortisol levels with resulting effects of hypercortisolism) when the drug was administered long-term. It has therefore been an obvious goal to develop antiprogestins that lack antiglucocorticoid activity. Lilopristone (ZK98.734) and onapristone (ZK98.299) (Fig. 3.1) reverse dexamethasone-induced tyrosine aminotransferase activity in rat hepatoma cells with roughly 5 and 4% the activity of mifepristone, respectively [24], and thus represent significant progress towards that goal. Indeed, as noted above, no other antiprogestins among the hundreds synthesized to date have been completely free of affinity to GR.

Lilopristone is very similar to mifepristone in structure, differing only in the substituent at the 17α position. This small modification appears to alter greatly its affinity to GR. It does not, however, seem to diminish the interaction of the compound with AAG, to which (like mifepristone) it appears to be highly bound in man [125]. Therefore the nonlinearities in pharmacokinetics and relatively long $t_{1/2}$ (presumably) associated with the saturable binding of mifepristone to AAG, may also be observed with lilopristone (no detailed clinical pharmacokinetic studies have yet been published for this compound).



Lilopristone Onapristone

Figure 3.1 Structures of the newer Schering antiprogestins; they are metabolized (like mifepristone) principally via N-demethylation of the 11β-dimethylaminophenyl moeity.

In contrast, onapristone possesses inverted stereochemistry at the 13 and 17 positions relative to lilo- and mifepristone, imparting different conformations to the C and D rings of the steroid nucleus and divergent 3-dimensional structure [46]. This may explain its apparent lack of binding to AAG in humans [125] and a resulting $t_{1/2}$ of only 2-4 hours [125]. Additionally, as described in Chapter 1, onapristone differs from mifepristone and lilopristone in that it may be devoid of any partial agonist activity (potentially related to differences in receptor phosphorylation [42, 43] and a lack of onapristone-receptor complex binding to DNA [39, 40]). This clearly could have important clinical implications.

Early work to evaluate the abortifacient potencies of lilopristone and onapristone in animals revealed that a dose of 1 mg/day *s.c.* (for 3 days) induced abortion in 4/4 rats treated with each of the Schering compounds, but in only 2/4 administered mifepristone [24, 45]. Experiments in the guinea pig produced similar results, in that 30 mg/day *s.c.* (for 2 days) of lilo- or onapristone induced abortion in 6/6 and 7/9 animals, respectively, while the same dose of mifepristone was effective in only 4/9 [24]. Of the two newer agents, however, only lilopristone has reportedly been used in a clinical trial for this indication. The overall rate of abortion in 96 women given 12.5, 25, 50 or 100 mg of the drug (orally, twice/day for 4 days) was only 68% [26]. This is similar to what is observed with mifepristone when administered alone; future trials should therefore evaluate coadministration with a prostaglandin analog. A clinical trial to evaluate the contraceptive potential of onapristone demonstrated that 15 or 50 mg/day (given during days 5-11 of the menstrual cycle), but not 5 mg/day, inhibited follicular development consistently without affecting subsequent luteal phase length, with no evidence of antiglucocorticoid activity [25]. These two newer agents thus appear to display similar antiprogestational activity to mifepristone *in vivo* in humans, although further trials are needed to ascertain whether their diminished antiglucocorticoid activities *in vitro* are indeed observed *in vivo*.

Lilopristone and onapristone thus represent very promising and potentially selective antiprogestational agents that, from the limited trials that have been performed, appear to be active *in vivo* and well tolerated. Their likely clinical use necessitates a better understanding of their metabolism. We hypothesized that despite their structural differences from mifepristone (which appear to alter significantly their binding to the glucocorticoid receptor and AAG (in the case of onapristone)), they are also metabolized by CYP3A4 due to the enzymes ability to accommodate very structurally diverse substrates.

3.3 Materials and Methods

3.3.1 Chemicals & specimens

Lilopristone, onapristone, their N-demethylated metabolites and gestodene were kindly supplied by Schering AG (Berlin, Germany). Diethyldithiocarbamic acid (DDC), NADPH, progesterone, quinidine, sulfaphenazole and troleandomycin (TAO) were purchased from Sigma Chemical Co. (St. Louis, MO). Furafylline was obtained from Research Biochemicals International (Natick, MA). Rabbit polyclonal antibodies specific to CYPs 3A4 and 2C9 were a generous gift of Dr. Steven A. Wrighton (Eli Lilly & Company, Indianapolis, IN). The microsomes used in the mifepristone CYP topology work (Chapter 2) were used for these studies; the liver donors were, again, a 53 year old male (HL-01), 5 year old male (HL-02) and 36 year old female (HL-03).

3.3.2 Assay for lilopristone, onapristone and their metabolites

The HPLC assay for the determination of mifepristone and its metabolites in serum [86], modified for measuring mifepristone and its metabolites in microsomal incubations, was adapted for measuring concentrations of lilopristone, onapristone and their metabolites. Briefly, the mobile phase was methanol:acetonitrile:water (35:30:35) but the

flow was reduced to 1.0 ml/min through a Zorbax C-18 (5μm x 4.6mm i.d. x 250mm) column with UV monitoring (315 nm). Under these conditions lilopristone, onapristone, their respective monodemethylated metabolites and progesterone (the internal standard) eluted with respective retention times of 13.3, 11.7, 7.6, 5.8 and 22.4 minutes. The autoinjector, pump and detector were Shimadzu models SIL-9A, LC-600 and SPD-6A, respectively. A Hewlett Packard 3392A integrator was used. Quantitation was effected with extinction coefficients from synthetic standards.

3.3.3 Incubation conditions

Incubations were carried out with 0.3 mg protein/ml in 0.1M Na_2HPO_4 buffer (pH 7.4) at 37°C, with substrate and inhibitors added in methanol (final concentration ≤2%, v/v). Following a 5 min pre-incubation period, reactions were initiated by adding NADPH in buffer (final concentration 1 mM), quenched after 2 min by adding a 2-fold volume of acetonitrile containing the internal standard and vortexed. Precipitated proteins were pelleted by centrifugation (5 min at 11,000g) and 100-150 μl of the supernatant subjected to HPLC analysis.

To evaluate mechanism-based or quasi-irreversible (TAO) inhibitors, catalysis dependent CYP inactivation, initiated by addition of NADPH, was carried out for 30 min, followed by ten-fold dilution of the microsomes with buffer containing substrate and NADPH. Thereafter, reactions were quenched at 2 min and samples processed as described above. In immunoinhibition experiments, various amounts of sera were incubated with microsomes at 24°C for 30 min before addition of substrate and the assay of catalytic activity. The antisera to CYP2C9 and CYP3A4 are maximally inhibitory at 75 and 200 μl/mg protein, respectively (S.A. Wrighton, personal communication).

3.3.4 Data analysis

As with mifepristone, kinetic parameters were estimated by non-linear regression analyses (Minim 3.0.8) assuming single enzyme Michaelis-Menten kinetics, with a weighting factor equal to the reciprocal of the observed initial rate. All results are presented as the means of duplicate determinations ± half the range.

3.4 Results

3.4.1 N-demethylation kinetics

Preliminary experiments with the two antihormones revealed the mono-N-demethylated derivatives to be their major metabolites in microsomal incubations, with smaller amounts of didemethylated metabolites detectable after extended incubation periods. Similar to mifepristone, we observed very short periods of linear product formation (≤3-4 min) and hence used 2 min incubations to insure conditions of linearity (data not shown). Figure 3.2A depicts a representative fit for lilopristone demethylation assuming single-enzyme Michaelis-Menten kinetics. Eadie-Hofstee transformation of the data (Fig. 3.2B) reveals a lack of biphasic kinetics at concentrations up to 200μM. This was also observed for onapristone demethylation (a representative fit is shown in Fig. 3.2C) and with the microsomes from the other liver donors. Table 3.1 summarizes the estimated kinetic parameters and the calculated intrinsic clearances (V_{max}/K_m) via demethylation for each compound. Rate of elimination (as assessed by the *in vitro* CL_{int}) was higher for lilopristone demethylation relative to that of onapristone with the exception of microsomes from HL-03. This was due to consistently lower apparent K_m for lilopristone in each of the microsomal samples relative to those for onapristone.

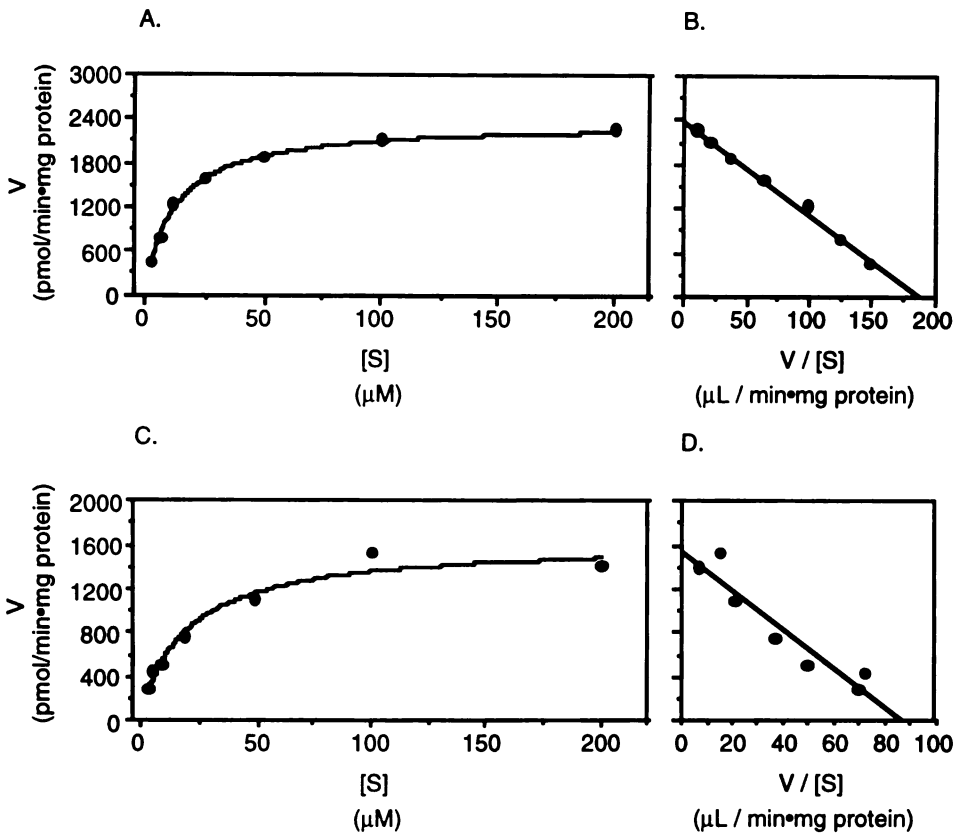


Figure 3.2 (A) Non-linear regression analysis for initial rate of lilopristone demethylation (for microsomes from HL-03) vs. substrate concentration assuming single-enzyme Michaelis-Menten kinetics and **(B)** Eadie-Hofstee transformation of the data. **(C)** Michaelis-Menten plot for onapristone demethylation in microsomes from HL-02. All data points represent the mean of demethylation rates determined in duplicate.

Table 3.1

Michaelis-Menten parameters for the demethylations of lilopristone and onapristone in human liver microsomes.

	Lilopristone			Onapristone		
	K_m	V_{max}	Cl_{int}	K_m	V_{max}	Cl_{int}
	μM	*pmol/min•mg protein*	*μl/min•mg protein*	*μM*	*pmol/min•mg protein*	*μl/min•mg protein*
HL-01	5.8	1270	219	20.2	1610	80
HL-02	9.9	2320	234	23.8	2770	116
HL-03	10.7	2310	216	14.8	4100	277
mean ± sd	8.8 ± 2.6	1970 ± 600	220 ± 10	19.6 ± 4.5	2830 ± 1250	160 ± 100

3.4.2 Effects of chemical inhibitors

Sulfaphenazole (up to 50μM) and quinidine (up to 25μM), competitive inhibitors of CYPs 2C9 and 2D6 respectively, had no effect on the initial rate of lilopristone demethylation upon coincubation with the substrate at 10μM (at or below its apparent K_m) (Fig. 3.3A). These two inhibitors used at 50 and 25 μM, respectively, also failed to inhibit onapristone demethylation (Fig. 3.3B). Similarly, mechanism-based inhibitors of CYP1A2 (furafylline) and CYPs 2A6 and 2E1 (DDC) did not inhibit either demethylation (Fig. 3.4B, Fig. 3.5) under conditions previously demonstrated to maximally inhibit their respective enzymes [114].

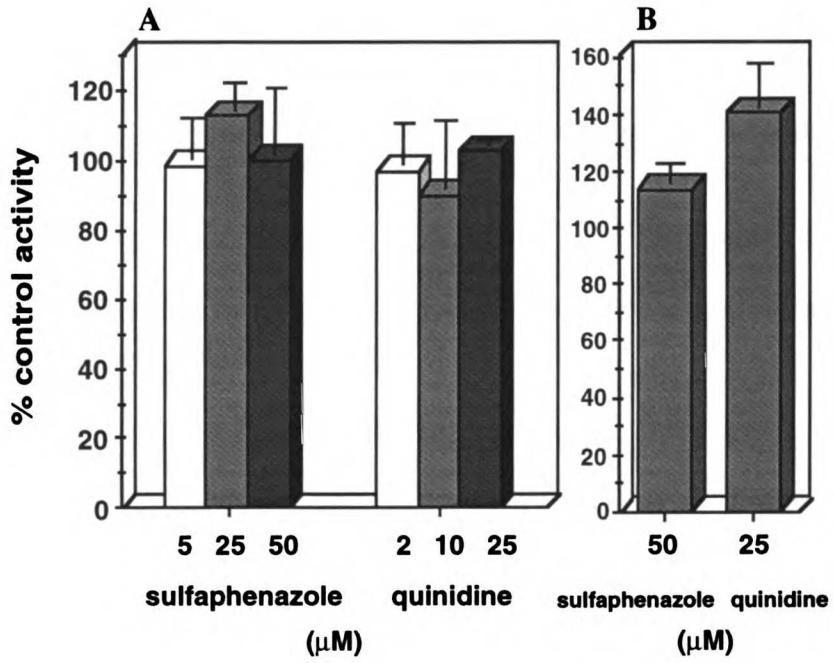


Figure 3.3 **(A)** Lack of inhibition of lilopristone demethylation in microsomes from HL-02 by competitive inhibitors of CYP2C9 (sulfaphenazole) and CYP2D6 (quinidine). **(B)** Lack of inhibition of onapristone demethylation by high concentrations of these two potent CYP inhibitors.

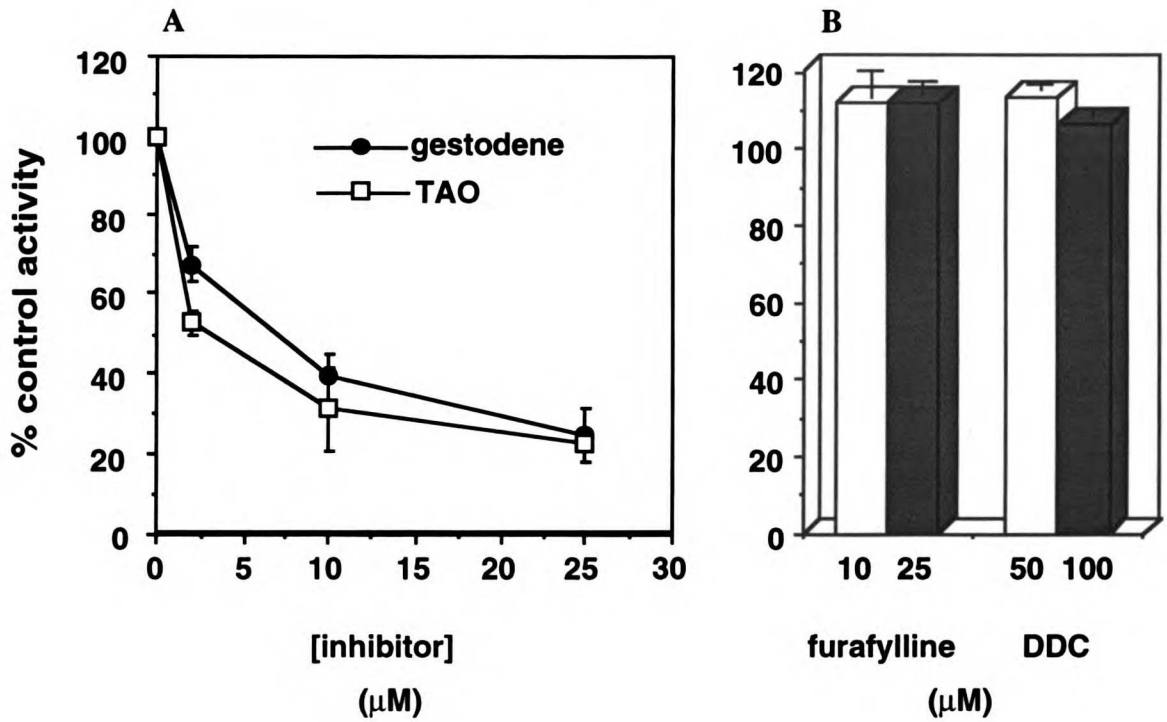


Fig. 3.4 **(A)** Inhibition of lilopristone demethylation by gestodene and TAO and **(B)** lack of inhibition by furafylline and DDC.

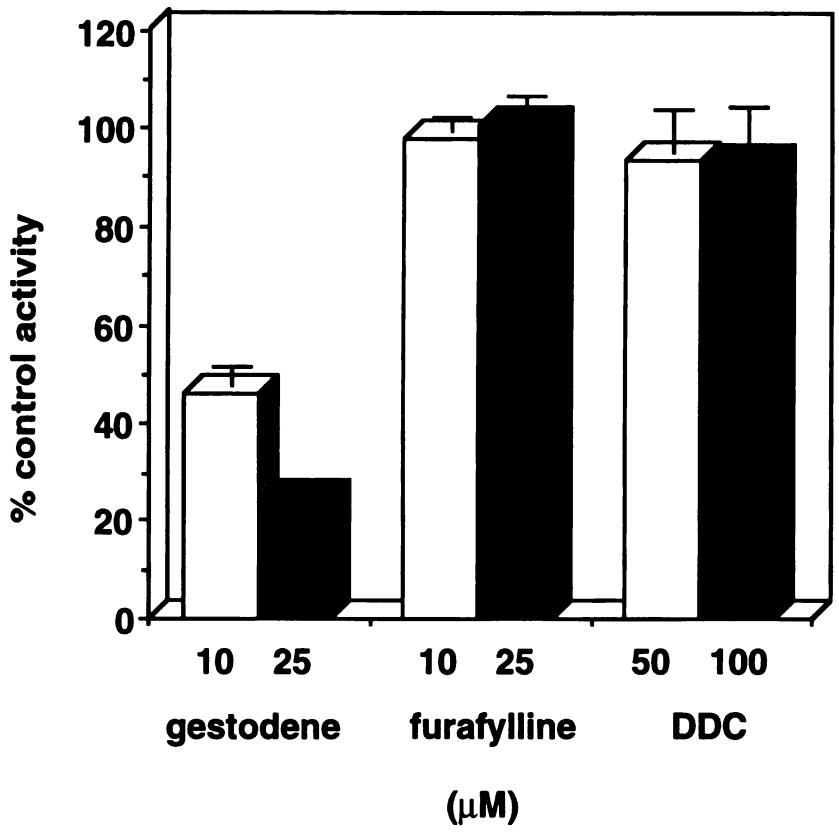


Figure 3.5 Effects of mechanism-based inhibitors of CYP3A enzymes (gestodene), CYP1A2 (furafylline) and CYPs 2A6 and 2E1 (DDC) on the initial rate of onapristone demethylation.

The CYP3A-selective inhibitors gestodene and TAO dose-dependently, potently and significantly reduced initial rates of lilopristone demethylation (Fig. 3.4A) with respective IC_{50} values of approximately 3 and 7μM. Gestodene at 10 and 25μM inhibited onapristone demethylation by 54 and 75%, respectively (Fig. 3.5), extents very similar to those observed for inhibition of lilopristone demethylation.

3.4.3 Effects of antibodies to CYPs 3A4 and 2C9

Antibodies to CYP3A4 were used at a concentration previously determined to maximally inhibit the enzyme and resulted in inhibition of lilopristone and onapristone demethylations by 70 and 82%, respectively (Fig. 3.6). Antibodies to CYP2C9 were also evaluated because of the aforementioned report (in Chapter 2) suggesting potential CYP2C subfamily involvement in mifepristone metabolism in the rat [87]. These antibodies did not inhibit either oxidation (Fig. 3.6) at a concentration known to maximally repress CYP2C9 catalyzed tolbutamide hydroxylation by 75% (S.A. Wrighton, personal communication), consistent with lack of inhibition by sulfaphenazole.

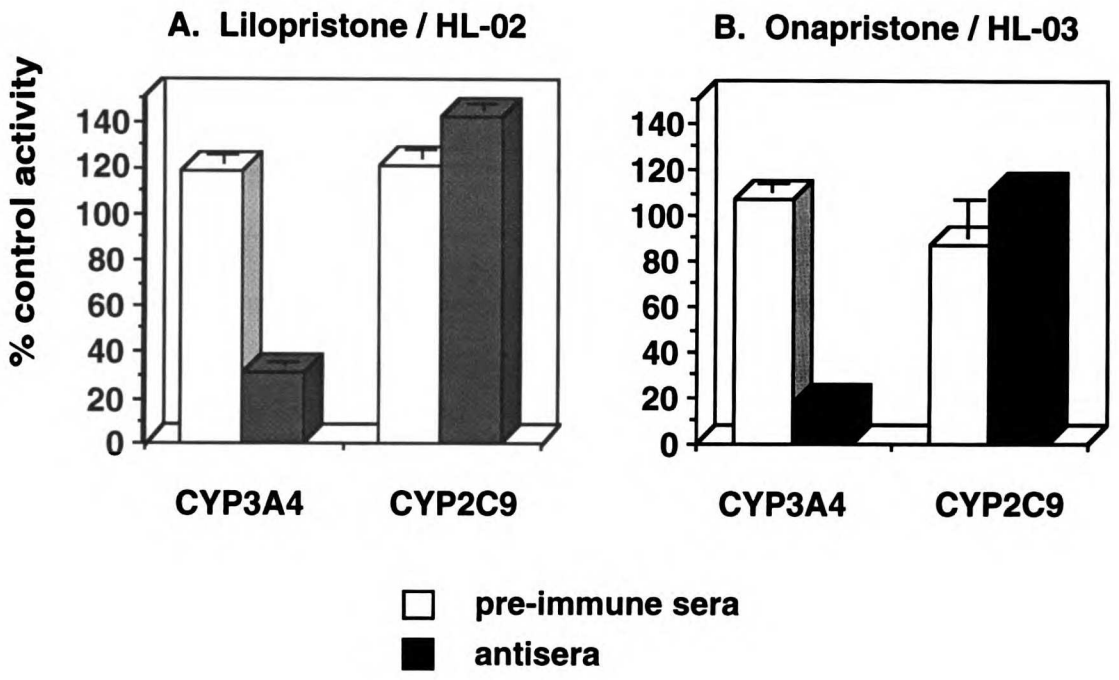


Figure 3.6 *Effects of antibodies to CYPs 3A4 and 2C9 on antiprogestin demethylations* Polyclonal antibodies to CYPs 3A4 and 2C9 (at 200 and 75 µl/mg microsomal protein, respectively) were incubated with microsomes from HL-02 **(A)** and HL-03 **(B)** for 30 min at 25°C to evaluate their effects on lilopristone and onapristone demethylations, respectively.

3.5 Discussion

In the present work, the collective data indicate that these two newer antiprogestins are metabolized primarily via N-demethylation in human liver microsomes and that CYP3A4 is the major enzyme catalyzing these reactions. To our knowledge, no data has been published descriptive of the *in vivo* biotransformations of these antihormones in humans. In experimental species, these two compounds are mainly metabolized by mono-N-demethylation [126]. Our *in vitro* results suggest that this is likely to be the major route of metabolism in humans as well. Unlike mifepristone, which is also metabolized via hydroxylation of its 17α-propynyl moiety, the respective 17α and 17β substituents of lilopristone and onapristone possess hydroxyl groups at their termini and, as might be expected, apparently do not undergo oxidation at these positions. The lack of biphasic kinetics for both antiprogestins in microsomes from each of the three liver donors suggests that a single enzyme is primarily involved in mediating their sole route of oxidative metabolism (N-demethylation). Additionally, it is extremely likely that their second demethylations are also CYP3A4 mediated, as we previously demonstrated for mifepristone [127].

Despite the structural differences from mifepristone that seem to influence several characteristics of these newer agents, the present study demonstrates that they are also principally metabolized by CYP3A4. Inhibition of these oxidations by gestodene, TAO and antibodies to CYP3A4 was maximal at roughly 70-80%, suggesting that other non-3A subfamily CYPs may catalyze the reactions as well but at a much lower rate (i.e., higher apparent K_m and lower V_{max} - resulting in the observed monophasic kinetics). This is likely for mifepristone as well. It is very probable, however, that CYP3A4 would be the clinically important site of potential drug-drug interactions due to its dominant role in the metabolism of the antihormones. This enzyme is now widely recognized to play an exceedingly important role in CYP-mediated xenobiotic metabolism. Furthermore, as we

recently hypothesized [128], coexpression of CYP3A enzymes and P-glycoprotein (P-gp) in the intestinal mucosa and in some tumor tissues likely plays an important role in influencing the oral bioavailability and chemotherapeutic efficacy, respectively, of agents that are combined CYP3A4/P-gp substrates. Mifepristone was recently shown to inhibit doxorubicin transport and azidopine photo-affinity labeling of P-gp [129], suggesting that the antihormone is a potential substrate and/or inhibitor of the transporter. Of interest for further study is whether these newer, promising antiprogestins are also P-gp substrates. In addition to their status as CYP3A4 substrates, this could markedly influence their oral bioavailabilities and efficacies as anticancer agents, and suggest another important locus of potential drug-drug interactions.

Chapter 4 ANTIPROGESTIN MEDIATED INACTIVATION OF CYP3A4

4.1 Summary

Based on previous observations of very short periods of linearity for antiprogestin metabolite formation and the presence of a common tertiary amine moiety in each compound that is the principal site of their metabolism, we hypothesized that mifepristone, lilopristone and onapristone are converted by CYP3A4 to reactive nitroso species that complex the heme of the enzyme, thereby inactivating it. Upon preincubation with human liver microsomes in the presence (but not the absence) of NADPH, mifepristone inhibits a marker of CYP3A4 catalytic activity, midazolam 1'-hydroxylation, very potently (IC_{50}~3.5μM) and extensively (by ~87%). Lilopristone and onapristone also display NADPH-dependent inactivation of CYP3A4 with characteristics very similar to mifepristone. However, utilizing human liver microsomes, we were unable to detect spectrophotometrically the metabolic-intermediates (which normally display absorbance maxima in the range of 445-455 nm) that are presumably formed upon complexation of the nitroso species to CYP3A4. Importantly, with these microsomes we were also unable to detect the metabolic-intermediate formed with troleandomycin, a quasi-irreversible inhibitor that in this respect is mechanistically prototypical. The observed lack of 445-455 nm absorbing complexes may thus be reflective of a deficiency in CYP3A forms in these microsomes relative to CYP3A-induced rat microsomes (in which virtually all such spectrophotometric studies have been performed). Therefore, antiprogestin mediated inactivation of CYP3A4 has been clearly demonstrated at concentrations attained *in vivo* (suggesting potential, significant drug-drug interactions), but the exact mechanism remains unconfirmed.

4.2 Introduction

In prior work investigating the metabolism of mifepristone, lilopristone and onapristone (Chapters 2&3), we observed very short periods of linear product formation (≤3-4 min), which was not affected by substitution of an NADPH regenerating system or higher concentrations of the cofactor. This led us to suspect that these compounds were being oxidized to reactive species capable of inactivating their metabolizing enzyme. Further strengthening this hypothesis is the presence of a tertiary amine (the 11β-dimethylaminophenyl substituent) in all three antiprogestins that is importantly also their principal site of metabolism.

Numerous investigators in the 1970s [130-137] demonstrated that secondary or tertiary amines could be dealkylated to the primary amine, which is then further oxidized to a reactive metabolic-intermediate (MI). This is thought to be a nitroso species that is capable of complexing the ferrous heme of the cytochrome P450 (CYP) which created it, thereby inactivating the enzyme (Fig. 4.1). Such metabolic-intermediate complexes normally display absorbance maxima in the range of 445-455 nm. Indeed, this is the

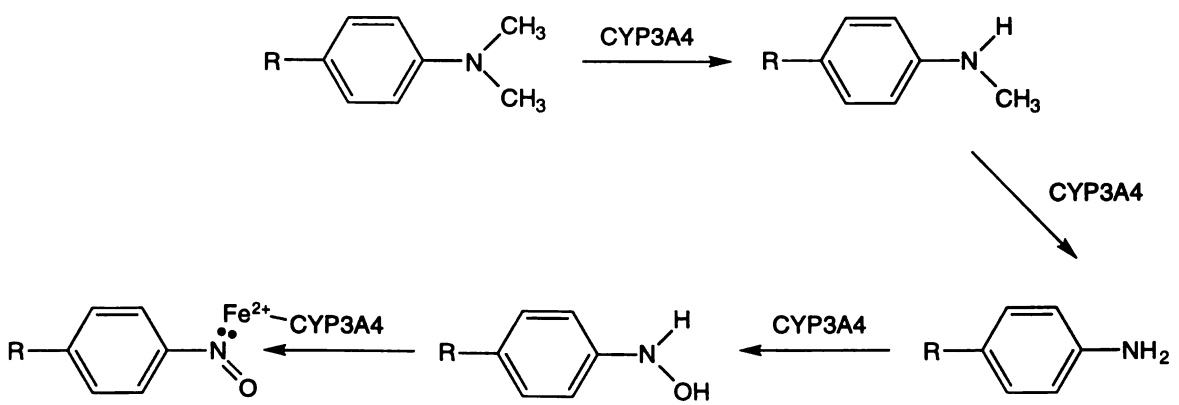
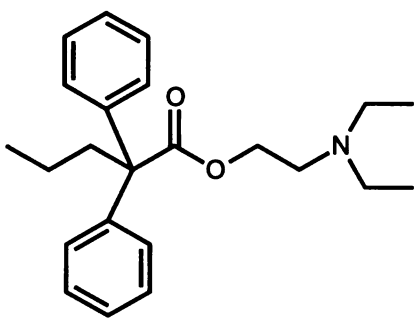


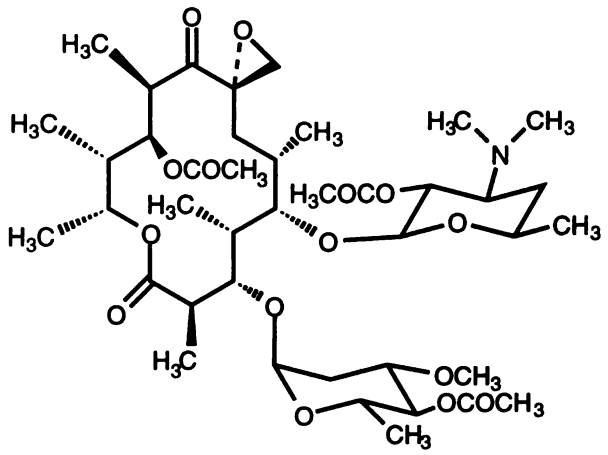
Figure 4.1 Mechanism of metabolic intermediate complex formation upon sequential oxidation of a tertiary amine (in this scheme the antiprogestin 11β-dimethylaminophenyl group) to the reactive nitroso species.

mechanism of inactivation by which SKF-525A (a relatively non-specific CYP inhibitor) and troleandomycin (which is selective for CYP3A enzymes and was used in work described in Chapters 2&3) quasi-irreversibly inhibit CYPs (Fig. 4.2). This type of inhibition is not mechanism-based (suicide) or true irreversible inhibition because it does not involve covalent modification of the heme or apoprotein and, in fact, can be reversed *in vitro* by the addition of potassium ferricyanide (which oxidizes the heme iron back to the ferric state, liberating active enzyme).

Because we have demonstrated a principal role of CYP3A4 in the N-demethylations of these antiprogestins, the aim of the work described in this chapter was to assess potential inactivation of CYP3A4 by these compounds in human liver microsomes. These findings could have important clinical implications, particularly for therapies involving repeated dosing or coadministration of the antihormones.



SKF-525A



TROLEANDOMYCIN

Figure 4.2 Structures of SKF-525A and troleandomycin, compounds possessing tertiary amine moeities that are oxidized to reactive nitroso species which complex the CYP heme (Fe^{2+}), leading to enzyme inactivation.

4.3 Materials and Methods

4.3.1 Chemicals & specimens

Mifepristone was a gift from Roussel Uclaf (Romainville, France). Lilopristone, onapristone, their N-demethylated metabolites and gestodene were kindly supplied by Schering AG (Berlin, Germany). Midazolam and its 1'-hydroxylated metabolite were gifts from Hoffmann-LaRoche (Nutley, NJ). Progesterone, flurazepam, NADPH, Tris-HCl, KCl, $MgCl_2$ and sodium phosphate were purchased from Sigma Chemical Co. (St. Louis, MO). HPLC grade methanol and acetonitrile were from Fisher Scientific (Pittsburgh, PA). For these studies, microsomes from HL-02 (from a 5 year old male) and HL-03 (from a 36 year old female) were used. Their preparation, protein and CYP measurements and storage conditions are described in Chapter 1 [138].

4.3.2 HPLC assays for midazolam, lilopristone, onapristone and their metabolites

The HPLC analysis of midazolam and its 1'-hydroxylated metabolite was carried out as previously described [138]. Briefly, the mobile phase was 10 mM potassium phosphate (pH 7.4):methanol:acetonitrile (44:35:21) at a flow rate of 1.0 ml/min through a Zorbax C-18 (5µm x 4.6mm i.d. x 250mm) column with UV monitoring (220nm). Under these conditions midazolam, 1'-hydroxymidazolam and flurazepam (the internal standard) eluted with respective retention times of 24.6, 11.5 and 36.5 minutes. The assay for measuring lilopristone, onapristone and their metabolites was performed as described in Chapter 3. For both assays, the autoinjector, pump and detector were Shimadzu models SIL-9A, LC-600 and SPD-6A, respectively, and a Hewlett Packard 3392A integrator was used. Quantitation was effected with extinction coefficients from synthetic standards.

4.3.3 Incubation conditions

To assess potential enzyme inactivation, 150 μg of HL-02 microsomal protein was preincubated at 37°C with each of the antiprogestins at various concentrations (added as methanolic solutions, final solvent concentrations ≤2%, v/v) for 5 min. NADPH (in 0.1M Na_2HPO_4 buffer, pH 7.4) or an equal volume of buffer was then added and incubations carried out for various lengths of time up to 30 min. Control incubations, to which the same volume of methanol was added, were incubated for the same time periods with 1 mM NADPH. At the end of these initial incubation periods, the microsomes were diluted 10-fold (final volume 500 μl) with the same buffer (at 37°C) containing NADPH and the marker substrate for CYP3A4 catalytic activity (20 μM midazolam, lilopristone or onapristone). Thereafter, reactions were quenched after 2 min (or 5 min for the measure of midazolam 1'-hydroxylase activity) by adding a 2-fold volume of acetonitrile containing the internal standard progesterone (or flurazepam for midazolam) and vortexed. Precipitated proteins were pelleted by centrifugation (5 min at 11,000g) and 100-150 μl of the supernatant subjected to the appropriate HPLC analysis. Results are presented as the means of duplicate determinations ± half the range.

4.3.4 Attempted spectrophotometric detection of 445-455nm absorbing complexes

Procedures were as previously described [139]. Briefly, microsomes from HL-02 or HL-03 were diluted to 2 mg protein/ml in 50 mM Tris-HCl buffer containing 150 mM KCl and 10 mM $MgCl_2$, divided into two cuvettes and a baseline recording initiated (scanning from 400-500 nm) with an Aminco DW-2 spectrophotometer in split-beam mode. After adding 2 mM NADPH to the sample cuvette, each of the antiprogestins or troleandomycin (as a positive control) were added in methanol (and an equal volume of the solvent added to the reference cuvette). The gain in absorbance was then monitored by scanning at various time points up to 1 hr.

4.4 Results

4.4.1 NADPH and time dependent inhibition of CYP3A4 activity

Mifepristone was found to very potently (IC_{50}~3.5 μM) and extensively (by ~87%) decrease the initial rate of midazolam 1'-hydroxylation when preincubated with the microsomes for 30 min (Fig. 4.3A). This inhibition was clearly NADPH dependent (Fig. 4.3B). The 10-fold dilution step prior to the assay of midazolam 1'hydroxylase activity appeared to minimize competitive inhibition by remaining antiprogestin concentrations (i.e., for complexation of CYP3A4 by 25 μM mifepristone, ≤2.5 μM would remain to competitively inhibit the 1'-hydroxylation of 20 μM midazolam).

Assessment of lilopristone and onapristone inactivation of CYP3A4 was performed using each antiprogestin as a marker substrate for measuring the inhibitory effects of the other. Lilopristone inhibits onapristone demethylation with very similar characteristics to mifepristone mediated inhibition of midazolam 1'-hydroxylation, wherein roughly 80% inhibition is observed with 25 μM inhibitor (Fig 4.4A). Onapristone similarly inhibits lilopristone demethylation with NADPH dependency (Fig. 4.4B). Also consistent with catalysis dependent inhibition, respective inhibition by lilopristone and onapristone was time dependent (Fig. 4.5).

4.4.2 Failure to detect 445-455 nm absorbing complexes

In human liver microsomes from both HL-02 and HL-03, and in three separate experiments, we were unable to detect 445-455 nm absorbing complexes with any of the three antiprogestins or with troleandomycin at concentrations ranging from 10-200 μM and for periods up to 1 hour (data not shown).

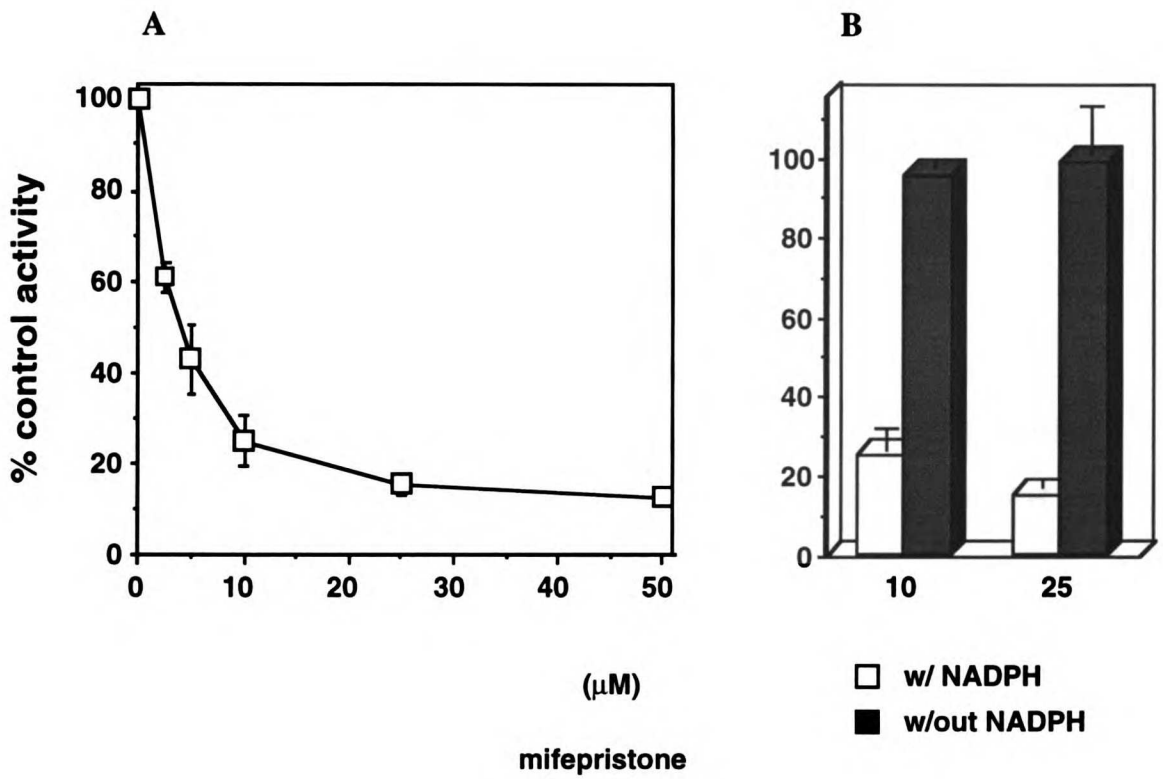


Figure 4.3 **(A)** Potent and extensive inhibition of midazolam 1'-hydroxylation in microsomes from HL-02 by mifepristone with a 30 min incubation or complexation period. **(B)** The inactivation is clearly NADPH dependent.

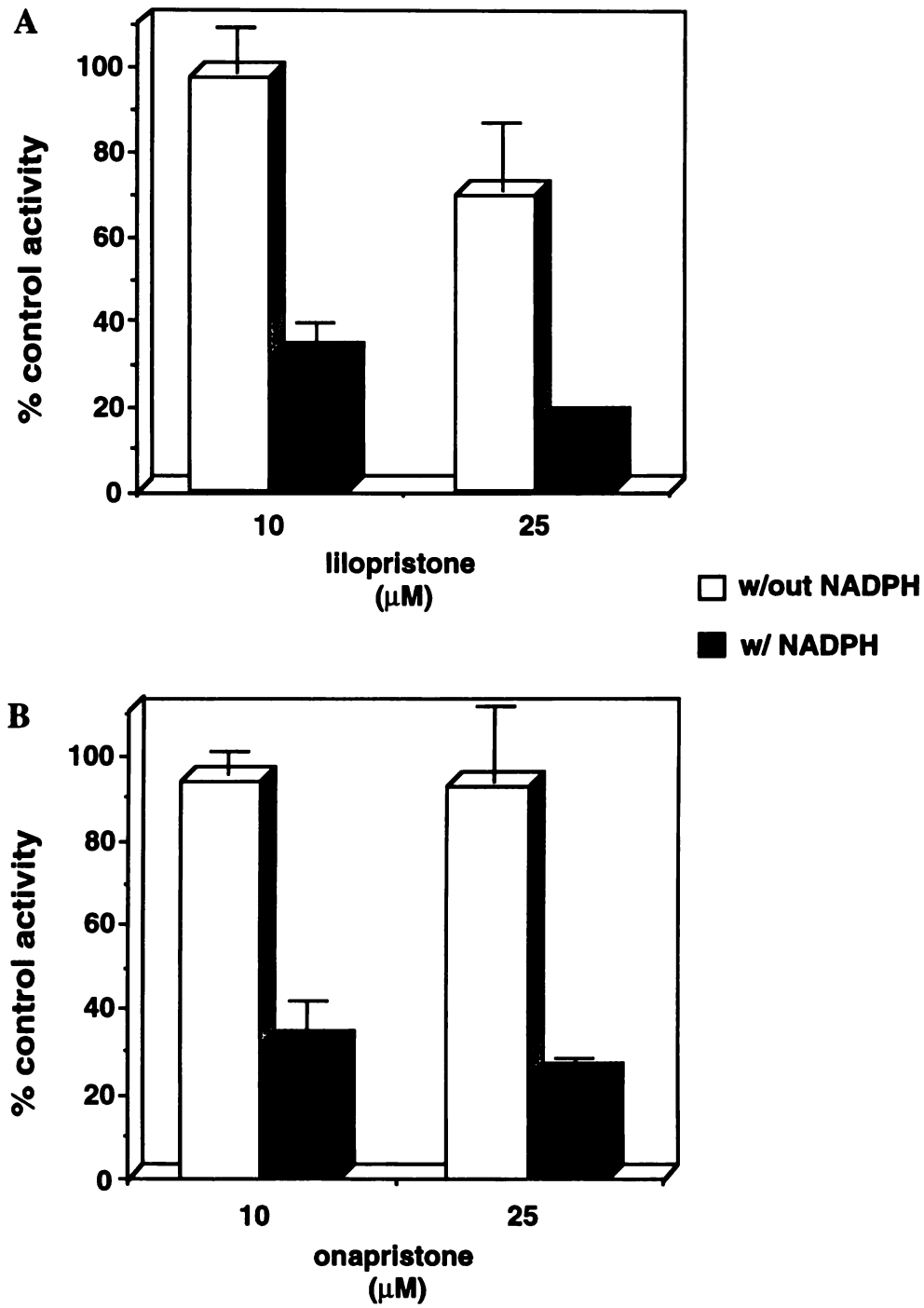


Figure 4.4 (A) Lilopristone mediated inhibition of onapristone demethylation in microsomes from HL-02 is extensive and NADPH dependent. (B) Onapristone inhibits lilopristone demethylation with very similar characteristics.

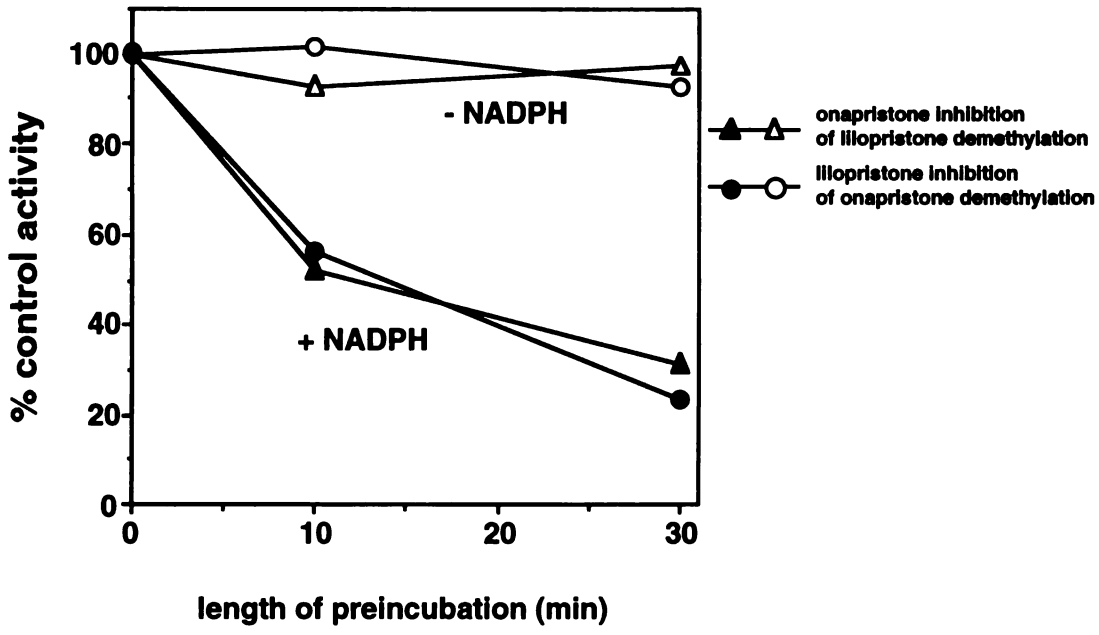


Figure 4.5 Time dependence of respective inhibition by 25 μM lilopristone and onapristone.

4.5 Discussion

Mifepristone was found to inhibit midazolam 1'-hydroxylation, a well established marker of CYP3A4 catalytic activity, in a manner consistent with inactivation of the enzyme. The inhibition was very potent, extensive and appeared maximal at 25 μM. Its potency and extent of inhibition are very similar to those we observed for gestodene and troleandomycin (Chapter 2) for their inhibition of mifepristone oxidations. Lilopristone and onapristone, which we observed in these microsomes to display similar apparent K_m for their N-demethylations relative to that of mifepristone (roughly 10-20 μM, Chapters 2&3), appear to inactivate CYP3A4 with similar characteristics under these conditions.

The two newer antiprogestins were used as respective markers of CYP3A4 catalytic activity to, in essence, simulate *in vitro* the effects the compounds would have on their own elimination if given chronically or in multiple doses. It is very likely that when sufficient doses of these antiprogestins are given orally, their concentrations in the intestinal lumen and liver during absorption exceed those observed later in plasma (which are roughly 2-4 μM for mifepristone at currently used doses) and therefore approach levels necessary to significantly inhibit CYP3A4. It thus seems reasonable to propose that oral administration of one of these antiprogestins (notably the 600 mg dose of mifepristone used in pregnancy termination) results in complexation and inactivation of a large fraction of CYP3A forms in both the intestine and liver. Clearly this would have more important clinical consequences upon multiple, extended administration of the antiprogestins for such uses as contraception or in multidrug therapy with other antineoplasts. In the former case, with subsequent doses the bioavailability and hepatic clearance of the antihormone may significantly increase and decrease, respectively, due to inactivation of CYP3A in the intestine and liver. Time-dependent changes in pharmacokinetics may thus be expected.

Antiprogestin mediated CYP3A4 inactivation may also be a particularly important consideration for their potential anticancer uses, since several antineoplasts such as taxol

[105], etoposide [106], vinblastine and other vinca alkaloids [107, 108] and tamoxifen [121] are known CYP3A4 substrates. Therefore multidrug therapies that incorporate one of these antiprogestins with other CYP3A4 substrates may result in increased toxicities (with time) associated with elevated drug concentrations. Conversely, the antiprogestins could have beneficial effects if, as we have hypothesized [128], CYP3A expression in tumor tissues modulates their response to anticancer agents that are substrates of the enzyme. Here inactivation of CYP3A in the neoplast would enable the cytotoxic effects of the antiprogestin and other agents to be realized.

As noted above (Chapter 1), mifepristone displays nonlinear pharmacokinetics characterized by a lack of dose-dependency for plasma levels and an unusual zero-order elimination phase of 24-48 hr for doses >50 mg. While saturable binding to α_1-acid glycoprotein seems to play a principal role in precipitating these phenomena, we hypothesize that the zero-order elimination phase observed for this drug is also due in part to inactivation of CYP3A4. Doses greater than 50 mg may result in extensive liver CYP3A4 complexation, drastically reducing elimination of the drug. After 24-48 hr, the the liver synthesizes more enzyme and significant elimination of the drug can occur.

In this work we were unable to detect 445-455 nm absorbing MI complexes for the antiprogestins. Although other metabolic pathways leading to reactive species cannot be excluded, the presence of the common 11β substituent in each antiprogestin and their similar characteristics of inhibition suggest a common mechanism involving the tertiary amine. We could also not detect the MI complexes for troleandomycin in these microsomes, indicating a potential, relative insufficiency in CYP3A levels in these samples for spectrophotometric detection. Indeed, nearly all such studies of MI complex formation have been performed using microsomes from rats treated with CYP3A inducing agents [130-137, 139-142].

In the most recent work [142], extensive 455 nm absorbing complexes were detected for SKF-525A in microsomes from dexamethasone or phenobarbital treated male

rats, but not in microsomes from control male animals (which do contain CYP3A forms constitutively). Among seven other compounds found to form easily detectable complexes with microsomes from dexamethasone induced rats, only L-α-acetylmethadol displayed detectable complex formation with the microsomes from control animals. The investigators were, however, able to demonstrate 455 nm absorbing complexes utilizing yeast-expressed human CYPs individually. Another study [143] has demonstrated measurable troleandomycin MI complex formation *in vivo* in humans, in microsomes from hepatic biopsies. The patients were given the drug for 7 days and total CYP was induced nearly two-fold (relative to microsomes from untreated controls). Complex formation *in vitro* with microsomes from the control patients was not assessed. It would thus appear that spectrophotometric detection of metabolic intermediate complexes depends greatly on the relative concentration of CYP3A forms in the microsomes used and the absorbance characteristics of the species.

Troleandomycin causes induction of CYP3A forms in both rats and man, primarily via protein stabilization. Therefore, although total and 3A subfamily CYPs are induced, the induced forms are complexed and thus inactive enzymes, and therefore do not result in increased CYP activity despite increased protein levels. In fact, in the above mentioned human study, antipyrine clearance was decreased by greater than 40% in the patients given troleandomycin for 7 days. By analogy, repeated antiprogestin administration may be expected to cause enzyme inactivation and induction through protein stabilization. The clinical significance of this probability also relies on whether these agents are able to induce CYP3A4 through transcriptional activation. Studies aimed at developing a system for the mechanistic study of this question are described in the chapter that follows.

Chapter 5 STUDIES TO EVALUATE POTENTIAL CYP3A4 REGULATION BY HORMONES AND ANTIHORMONES

5.1 Summary

The human hepatoblastoma cell lines HepG2 and Huh-7 were transiently transfected with a chimeric DNA construct consisting of a 1.2 kb fragment of the CYP3A4 5'-flanking region, the SV40 promoter and the firefly luciferase reporter gene to evaluate potential steroid hormonal and antihormonal transcriptional activation of the CYP3A4 regulatory region. Drugs that are known to induce transcription of CYP3A4, such as dexamethasone, rifampicin and phenobarbital, failed to induce expression of the construct in both cell types. Attempts to induce the glucocorticoid receptor in these cells had no effect on their response to inducing agents. Cotransfection of an expression vector for the rat glucocorticoid receptor in HepG2 resulted in moderate dexamethasone mediated luciferase induction, which was greatly enhanced by also cotransfecting an expression vector for the DNA-binding domain of the receptor. This latter result was dependent on the precise ratio of the cotransfected receptor and DNA-binding domain expression vectors and was very reproducible. The optimal plasmid ratio resulted in only a small, albeit significant response to mifepristone, but not to lilopristone or onapristone. It is concluded that these two cell lines are not suitable for CYP3A4 regulation studies involving transient transfection of promoter constructs because of their lack of response to well-established CYP3A4 inducing agents and the difficulty in interpreting the relevance of the induction observed with cotransfected glucocorticoid receptor and related expression vectors.

5.2 Introduction

The overall goal of the work described in this chapter was to evaluate two human hepatoma cell lines, HepG2 and Huh-7, for their suitabilities in the study of potential CYP3A4 transcriptional activation by steroidal hormones and antihormones. These studies were conducted in collaboration with the laboratory of Dr. Svein Oie, utilizing transient transfection of chimeric DNA constructs consisting of 1.2 kilobases of the *CYP3A4* 5'-flanking or regulatory region, a heterologous (SV40) promoter and an easily assayed reporter gene (firefly luciferase). The work was initiated primarily due to the clinical importance of potential CYP3A4 induction by these antihormones subsequent to their long-term administration.

For our purposes there did not exist an appropriate animal model or *in vitro* system in which to perform these studies. Importantly, there are significant species differences in the regulation of cytochromes P450, particularly those enzymes of the 3A subfamily. In rats, for example, males constitutively express relatively high but females extremely low levels of CYP3A forms in the liver [144] (the opposite trend may be true in humans, as discussed below). Rifampicin and pregnenolone 16α-carbonitrile, two effective CYP3A inducers in humans, do not induce these forms in the rat or rabbit, respectively [145]. Therefore cultured hepatocyte or *in vivo* studies (the latter are also prohibitive due to limited amounts of the antiprogestins) with an experimental species would perhaps yield results not applicable to humans. A potentially valid model, human hepatocytes in primary culture, was not feasible due to extreme difficulty in not only acquiring sufficient and frequent human liver specimens, but also in procuring tissue suitable for the successful isolation and culture of hepatocytes. Therefore human hepatoma cell lines potentially provided a more appropriate and easily maintained model system for this work. Furthermore, by utilizing transfected reporter plasmids in these cell lines, it was anticipated that mutations in the *CYP3A4* upstream region could later be performed to allow more detailed exploration of

the sequences and transcription factors involved. Cotransfection of expression vectors for the steroid receptors might also allow elucidation of receptor involvement. Therefore, one important advantage of this system was its potential to yield much more detailed information about the mechanisms involved in CYP3A4 transcriptional activation.

We hypothesized that these antiprogestational agents could potentially induce CYP3A4 not only via protein stabilization (as discussed in Chapter 4), but also through transcriptional activation (i.e., increased mRNA and protein synthesis). Transcription of CYP3A4 is activated by several synthetic steroidal agents, most notably dexamethasone (a glucocorticoid), pregnenolone 16α-carbonitrile (an antiglucocorticoid, noted above) and tamoxifen (an antiestrogen). We further theorized that if compounds such as dexamethasone and pregnenolone induce CYP3A4 via interactions with the glucocorticoid receptor, then mifepristone but not lilopristone or onapristone (due to their lower affinities to this receptor) may induce the protein through a similar mechanism. Importantly, the 5'-flanking region of CYP3A4 contains a progesterone/glucocorticoid response element and 3 estrogen response elements, as well as several other potentially important regulatory elements ([146], Fig. 5.1). However, Schuetz et al. [147] have proposed a 'non-classical' mechanism involving the glucocorticoid receptor for dexamethasone mediated induction of CYP3A1 in the rat. This and other, more recent work suggest the involvement of 'novel' response elements in CYP3A4 upregulation.

Potential modulation of CYP3A4 expression by antiprogestins may also be supported by its possible regulation by endogenous steroids. As recently reviewed by Harris, Benet and Schwartz [148], there are several lines of evidence that suggest a gender difference in CYP3A4 expression and/or activity, which we further hypothesize may be related to gender differences in steroid hormone levels. Liver microsomes from females were found to metabolize erythromycin, a well-characterized substrate of CYP3A4, at a rate roughly 25% greater than that observed with microsomes from male liver [149]. These *in vitro* findings appear to be supported by *in vivo* studies that have reported higher

erythromycin clearances [150] and higher rates of its N-demethylation (as measured by the erythromycin breath test) [151] in women versus men. Other studies have indicated greater rates of elimination in women for cyclosporine [152], alfentanil [153], prednisone and methylprednisolone [154, 155], tirilazad [156], verapamil [157] and diazepam [158] (all compounds known to be either principally or partly metabolized by CYP3A4). However, some studies have found no gender difference in the clearances of other known CYP3A4 substrates [159, 160] or in the levels of the enzyme in banks of liver microsomes [89, 161]. As noted in the Harris et al. review [148], these latter studies may have been confounded by inherently large interindividual differences in CYP3A4 expression, inadequate sample sizes, concomitant medications or, in the microsomal analyses, diseased liver samples. Interestingly, consistent with hormonal regulation of the enzyme, an observed decrease in alfentanil clearance with age in women (but not men) may correlate with menopausal status (i.e., a decrease with menopause) [153, 162]. In our laboratory, studies have demonstrated a similar trend for prednisolone [163] and erythromycin [164], and slight (but not statistically significant) increases in the clearances of both drugs with progesterone replacement therapy [164]. Data indicating steroidal regulation of CYP3A4 are thus inconclusive. Development of a suitable cell culture system would allow direct evaluation of not only antihormone mediated, but also endogenous steroid mediated transcriptional activation of the enzyme.

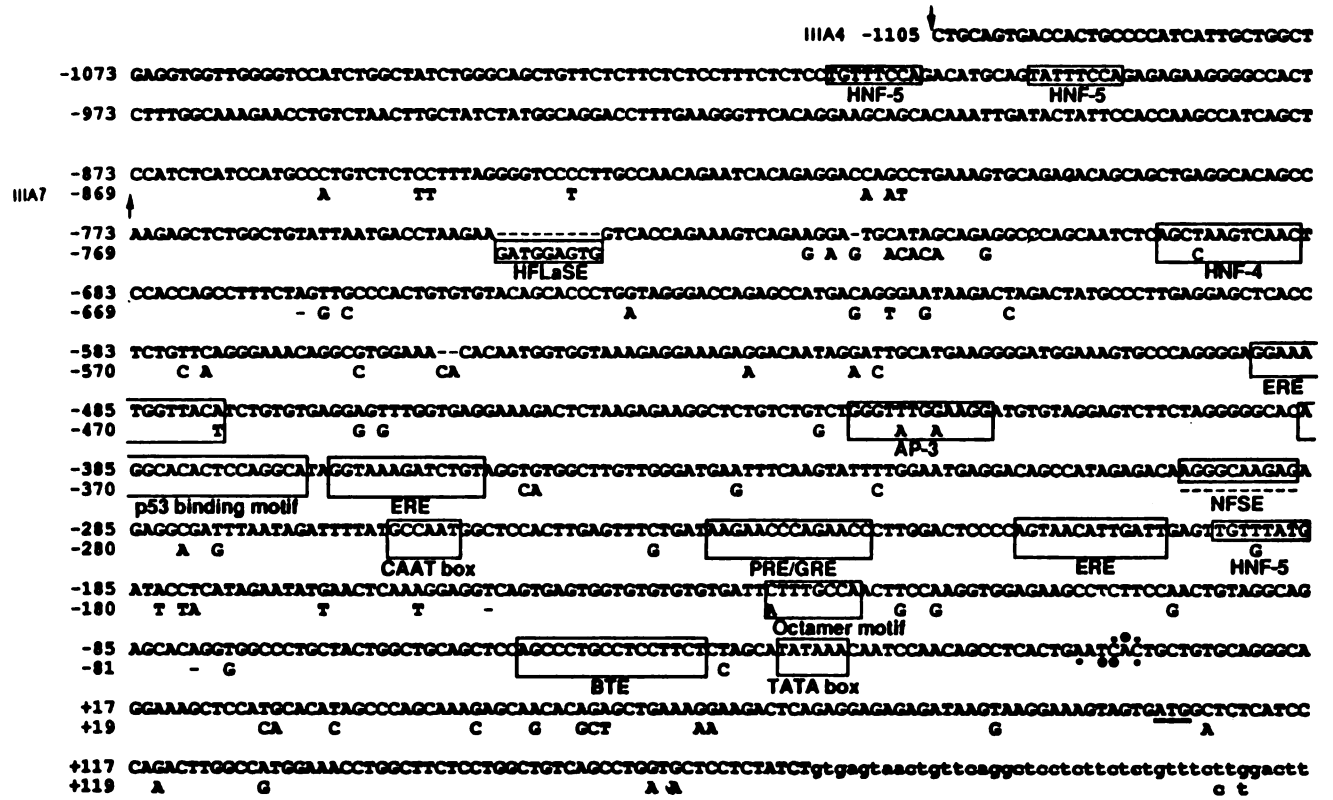


Figure 5.1 Nucleotide sequences of the CYP3A4 (upper) and CYP3A7 (lower) 5'-flanking regions [146]. Putative binding sites for known transcription factors are boxed. HNF-4 and -5, hepatic nuclear factors-4 and -5; NFSE, CYP3A4 (P450NF)-specific element; HFLaSE, CYP3A7 (P450HFLa)-specific element; PRE/GRE, progesterone/glucocorticoid response element; ERE, estrogen response element; BTE, basic transcription element. Large and small circles (in the -4/+2 region) indicate major and minor transcription initiation sites, respectively.

5.3 Materials and Methods

5.3.1 Chemicals and tissue culture/molecular biological reagents

Mifepristone, lilopristone and onapristone were obtained as noted in Chapters 2 & 3. Dexamethasone, phenobarbital, rifampicin, progesterone, hydrocortisone and dimethyl-sulfoxide were purchased from Sigma Chemical Co. (St. Louis, MO). LipofectAmine and DH5α *E. Coli* were obtained from Gibco BRL (Gaithersburg, MD). Wizard Mini- and Maxiprep DNA purification systems, pT7Blue and pGL2 vectors, restriction enzymes, ONPG, luciferase assay reagent and β-galactosidase were purchased from Promega (Madison, WI). Qiagen Endotoxin-free plasmid preparation kits were from Qiagen Corp. (Chatsworth, CA). Growth media, phosphate buffered saline, trypsin, heat-inactivated fetal bovine serum (FBS), HEPES and penicillin/streptomycin were from the UCSF Cell Culture Facility. Falcon tissue culture flasks (75 cm^2, vented cap), 12-well plates and all other tissue culture supplies were purchased from Fisher Scientific (Pittsburgh, PA).

5.3.2 Cell lines

HepG2 and Huh-7 hepatoblastoma cell lines were purchased from American Type Culture Collection (Rockville, MD). HepG2 were maintained in α-MEM supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. Huh-7 were cultured in DME H-21 with 10 μM HEPES and the same concentrations of FBS, penicillin and streptomycin as indicated for HepG2 cells.

5.3.3 Isolation of 1.2kb of the CYP3A4 upstream region (CYP3A4(-1105/+66))

Work briefly described in this and the following section followed standard protocols and was performed by members of Dr. Oie's laboratory. Human genomic DNA was isolated from blood using a GIBCO genomic DNA isolation system (GIBCO BRL) following the manufacturers instructions. Amplification and isolation of approximately 1.2

kilobases of the CYP3A4 5'-flanking region was performed by polymerase chain reaction (PCR), following standard protocols, with primers designed to distinguish CYP3A4 from CYP3A7. Primers corresponded to nucleotides (-1105 to -1087) and (+66 to +46) relative to the transcription initiation site (Fig. 5.1). The PCR product was isolated and purified by 2% agarose gel electrophoresis, β-agarase digestion and the Wizard PCR Prep system (Promega). The purified product was then cloned into pT7Blue vectors and the resulting plasmids were used to transform Nova Blue *E. Coli*. Plasmids were then isolated and purified from overnight cultures of successful recombinants using Wizard Maxiprep DNA purification kits. Restriction analyses (with the enzymes *Pst* I and *Sac* I) were carried out and sequencing was performed by the UCSF Biomolecular Resource Center to confirm a correct CYP3A4(-1105/+66) insert.

5.3.4 Cloning of CYP3A4(-1105/+66) into the pGL2 promoter vector

CYP3A4(-1105/+66) was excised from the pT7Blue vector using *Xba* I and *Bam*H I, and isolated and purified using standard protocols as described above. This was ligated into the pGL2 vector (Fig. 5.2A), which contains a simian virus promoter (SV40) and the firefly luciferase gene (LUC), that had been cut with *Nhe* I and *Bgl* II to generate ends compatible to those of the insert. Competent DH5α *E. Coli* were then transformed, recombinants grown and the resulting CYP3A4(-1105/+66)SV40-LUC plasmid (Fig. 5.2B) isolated and purified (Wizard Maxiprep kits). Restriction analysis (*Sac* I) verified the presence of the correct insert.

5.3.5 Rat glucocorticoid receptor (GR) and other expression vectors

The rat GR expression vector 6rRSVrGR and a vector for the GR DNA-binding domain, 6rRSVrGR(407-525), were kind gifts of Dr. Keith Yamamoto (UCSF Department of Cellular and Molecular Pharmacology). Transformation of DH5α *E. Coli* was performed, recombinants cultured and plasmids purified using a Qiagen Endotoxin-free

plasmid preparation kit. Fidelity of the expression vectors was confirmed by restriction analysis (*Bam*H I). The plasmid used as an internal standard (to correct for variable transfection efficiencies), pCMVβ, consisted of the β-galactosidase gene and the cytomegalovirus immediate early promoter, and was a kind gift of Dr. Frank Szoka (originally obtained from the Howard Hughes Medical Institute).

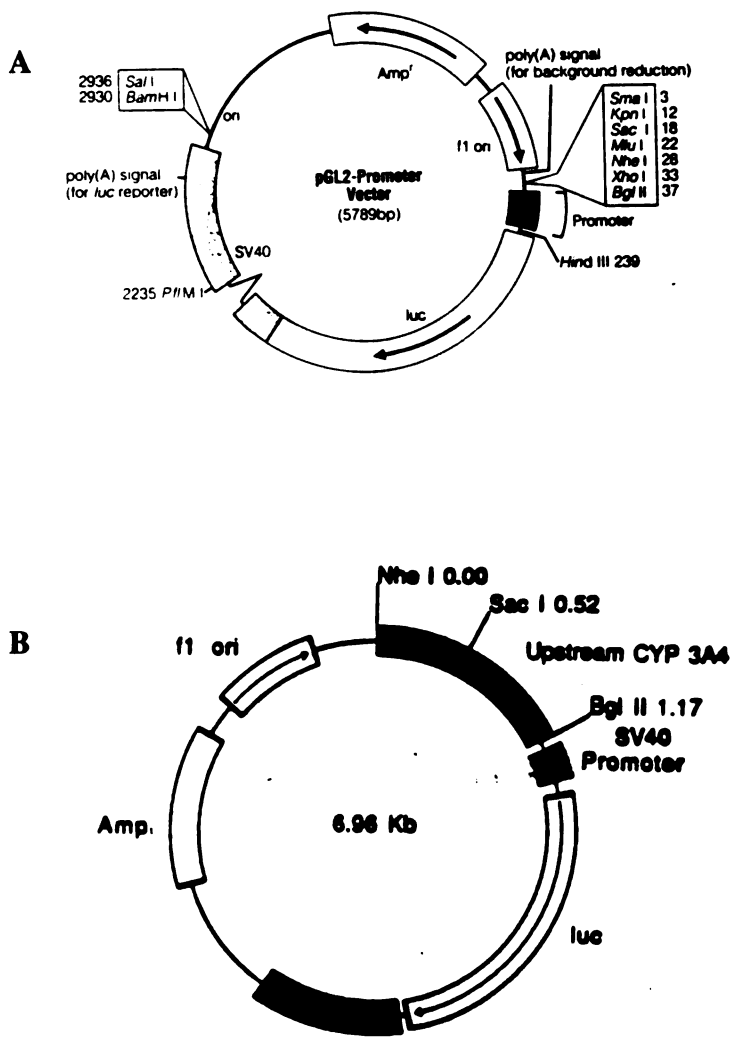


Figure 5.2 (A) The pGL2 promoter vector and **(B)** the construct, CYP3A4(-1105/ +66)SV40-LUC, used to transfect HepG2 and Huh-7 cells.

5.3.6 Cell culture and transfections

HepG2 and Huh-7 were maintained at 37°C in a humidified atmosphere of 95% air, 5% CO_2. Both cell types were subcultured by trypsinization and 4-6x10^5 HepG2 and 5-8x10^4 Huh-7 cells seeded per well (12-well plates) in 1 mL of the appropriate growth media, 18-24 hr prior to transfections. In initial experiments, cells were transfected at 60-80% confluence in serum and antibiotic-free media using the cationic lipid mixture LipofectAmine and 0.5 μg of the CYP3A4(-1105/+66)SV40-LUC plasmid, following the instructions of the manufacturer. After a 5 hr transfection period, an equal volume of media containing 20% FBS was added to the wells. At 18-24 hr after the start of transfection, the media was replaced with complete growth media and drug treatments initiated. In later work, transfections were affected via standard calcium phosphate co-precipitation (with cells at 40-60% confluence) of 1.0 μg CYP3A4(-1105/+66)SV40-LUC and 0.1 μg of the pCMVβ internal standard plasmid, with or without various amounts of 6rRSVrGR and/or 6rRSVrGR(407-525). After a 6 hr transfection, cells were rinsed twice with PBS and complete media added. Following both transfection methods, drug treatment was carried out for 12-72 hours with the various compounds added as 1000-fold stock solutions in DMSO (i.e., final DMSO concentration was 0.1%). Control wells received the same volume of DMSO. For treatment periods greater than 24 hr, media was replaced daily with that containing freshly added test compound. All transfections and drug treatments were performed in triplicate and results are presented as the means ± sd (except as noted in Fig. 5.8).

5.3.7 Cell Harvest

Following treatment periods, cells were rinsed twice with PBS and lysed by adding 150 μl of cell lysis buffer (250 mM Tris-HCl, pH 8.0, 0.1% Triton X-100). After 15 min incubation at room temperature, cells were harvested by scraping and then transferred to microcentrifuge tubes kept on ice. Lysates were then frozen in a dry ice/acetone bath, to

insure complete lysis, and thawed at room temperature. These lysates were then vortexed for 15 sec and cell debris pelleted by centrifugation (40-45 sec @ 11000g). The supernatants were then transferred to new tubes and stored at -80°C or kept on ice (if the reporter gene assays were to be performed immediately).

5.3.8 Luciferase and β-galactosidase assays

The assay of firefly luciferase activity in cell extracts was performed using Promega luciferase assay reagent following the instructions of the manufacturer. The assay measures light production (photon release) from the luciferase mediated, ATP-dependent oxidation of beetle luciferin. Briefly, 100 µl of the assay reagent (20 mM tricine, 1.07 mM [$(MgCO_3)_4$ $Mg(OH)_2$ x 5 H_2O)], 2.67 mM $MgSO_4$, 0.1 mM EDTA, 33.3 mM DTT, 270 µM luciferin, 530 µM ATP, pH 7.8) was added to 20 µl of cell extract (both thawed to room temperature) in a luminometer and light production measured and integrated over a 10 sec interval. Values are expressed as relative light units (RLU) normalized to either total cell extract protein (measured by the Pierce bicinchoninic assay) or to β-galactosidase activity.

β-Galactosidase activity (cleavage of o-nitrophenyl-β-D-galactopyranoside, ONPG) was measured using a microtitre, photometric assay. Briefly, 50 µl PBS containing 0.5% FBS and 50 µl cell extract were added to each well, with 50 µl cell lysis buffer used in place of cell extract for wells containing the β-gal standards (16 serial dilutions of 2 units of the enzyme). The substrate solution was 2 mg/ml ONPG in assay buffer (60 mM Na_2HPO_4, pH 8.0, 1 mM $MgSO_4$, 10 mM KCl, 50 mM β-mercaptoethanol). A 150 µl aliquot of this solution was added per well and incubations at room temperature were carried out for 10-30 min, depending on visual assessment of enzyme activity. Optical density (410 nm) was read on a Photodyne plate reader.

5.4 Results

5.4.1 Initial results with HepG2

Preliminary experiments utilizing Lipofectamine mediated transient transfection of HepG2 cells demonstrated optimal transfection efficiencies and cell viabilities with 2 µl of the cationic lipid mixture and 0.5 µg reporter plasmid per well, and a 5 hr transfection period (data not shown). Cells transfected under these conditions were then treated with two known CYP3A4 inducers, dexamethasone (DEX) and rifampicin (RIF), and mifepristone (MIF) (all 10 µM) for 48 hr. The two known inducers failed to significantly activate luciferase expression, whereas mifepristone did so by roughly 45% ($p<0.05$) (Fig. 5.3A). Treatment of the cells for 48 hr with higher concentrations of these compounds and with 50 µM troleandomycin (TAO) and 10 µM progesterone was then evaluated. DEX at the higher concentration (50 µM) induced transcription by roughly 50% (although this was not significant) (Fig. 5.3B). Mifepristone at these concentrations lowered cell viability substantially, leading to drastic reductions in luciferase activity (despite protein normalization) (Fig. 5.3B). 50 µM RIF and TAO (which, as noted in Chapter 4, induces CYP3A forms primarily via post-translational mechanisms and was here included somewhat optimistically as a negative control) did not induce luciferase expression, nor did 10 µM progesterone (a supraphysiologic concentration even for pregnant women) (Fig. 5.3B). These results were obtained with compounds added to complete growth media. Because FBS contains cortisol and other hormones that could potentially interfere with an induction response, exclusion of this component was then evaluated. This had no significant effect on potential MIF or phenobarbital mediated transactivation, but did significantly increase the response to DEX ($p<0.01$), but only to 160% of control values and with significant effects on cell growth (Fig 5.3C).

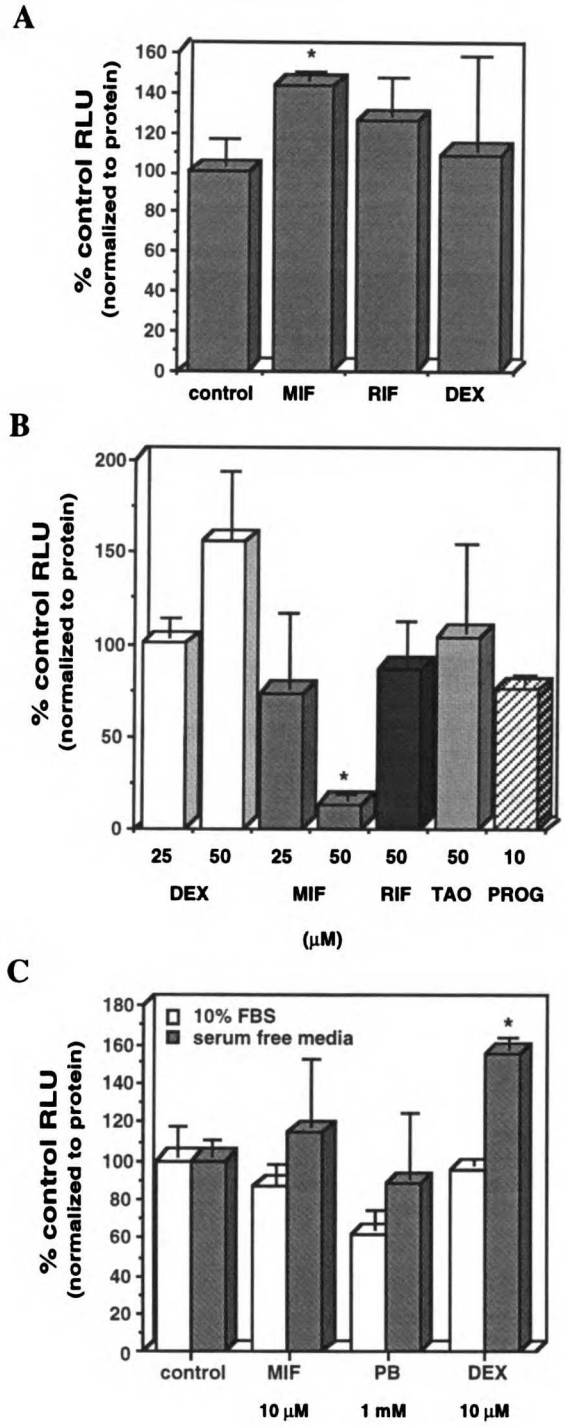


Figure 5.3 **(A)** Effects of 48 hr treatment with 10 μM mifepristone (MIF), rifampicin (RIF) and dexamethasone (DEX) on transiently transfected construct expression in HepG2 cells. **(B)** Effects of higher concentrations of these compounds, troleandomycin (TAO) and progesterone (PROG). **(C)** Effects of drug treatment in serum free media (PB, phenobarbital). * P<0.01 relative to control.

5.4.2 HepG2 and Huh-7 transfections with β-gal internal standard

Cell lines cultured for extended periods may lose certain differentiated functions and may also differ depending upon their source. For these reasons, new HepG2 were ordered from ATCC. Additionally, experiments with the second cell line Huh-7 were initiated. In preliminary experiments using standard calcium phosphate coprecipitation, and with both cell types, very good levels of reporter gene activity were obtained with 1.0 μg CYP3A4(-1105/+66)SV40-LUC, 0.1 μg pCMVβ and a 6 hr transfection period (data not shown).

Treatment of both cell types following transfection with 20 μM DEX, RIF, each of the three antiprogestins, and a combination of DEX and hydrocortisone (HC) for 48 hr did not induce luciferase expression (Fig. 5.4A & B). The last treatment was evaluated due to a report that HC induces GR in Huh-7, an effect that was potentially steroid-specific and that we thought could perhaps compensate for lack of induction resulting from insufficient GR expression in the cells. Treating the cells first for 24 hr with HC, to potentially induce GR independently of the effects of the other compounds, followed by 24 hr treatment with DEX, MIF, lilopristone (LILO) or onapristone (ONA) failed to induce luciferase in Huh-7 (Fig. 5.5B) and caused a uniform reduction in normalized luciferase activity in HepG2 (Fig. 5.5A).

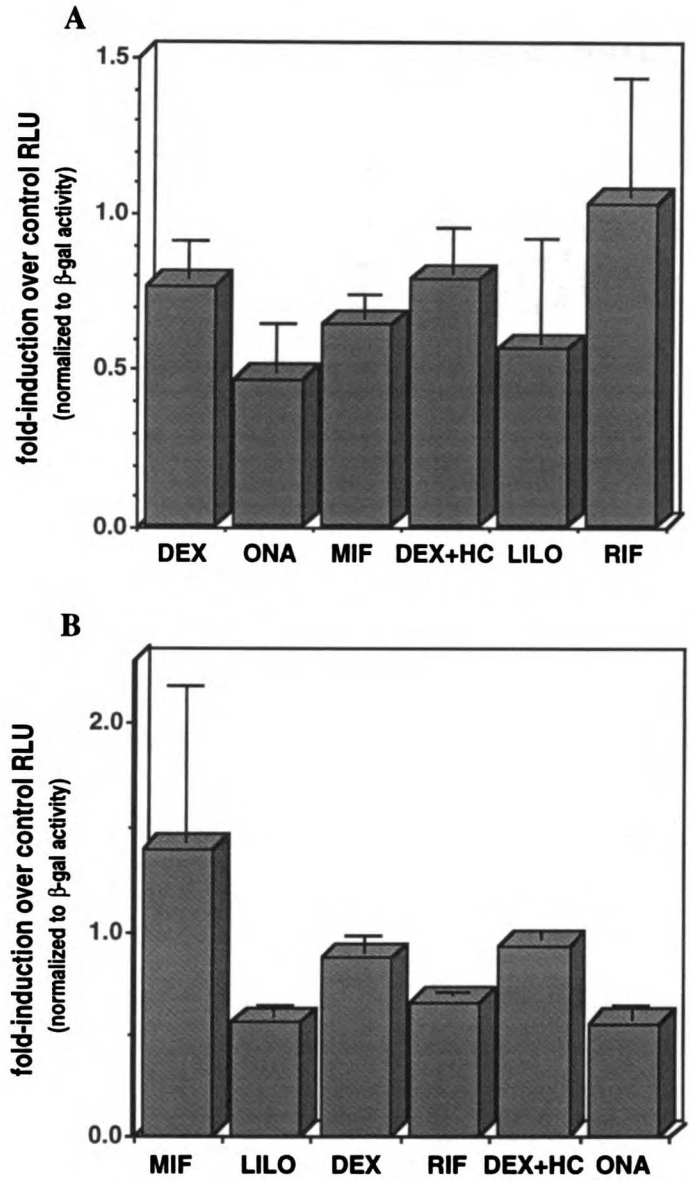


Figure 5.4 Effects of 20 μM antiprogestins and known CYP3A4 inducers on construct expression (luciferase activity normalized to that of β-galactosidase) in **(A)** HepG2 and **(B)** Huh-7 cells treated for 48 hr.

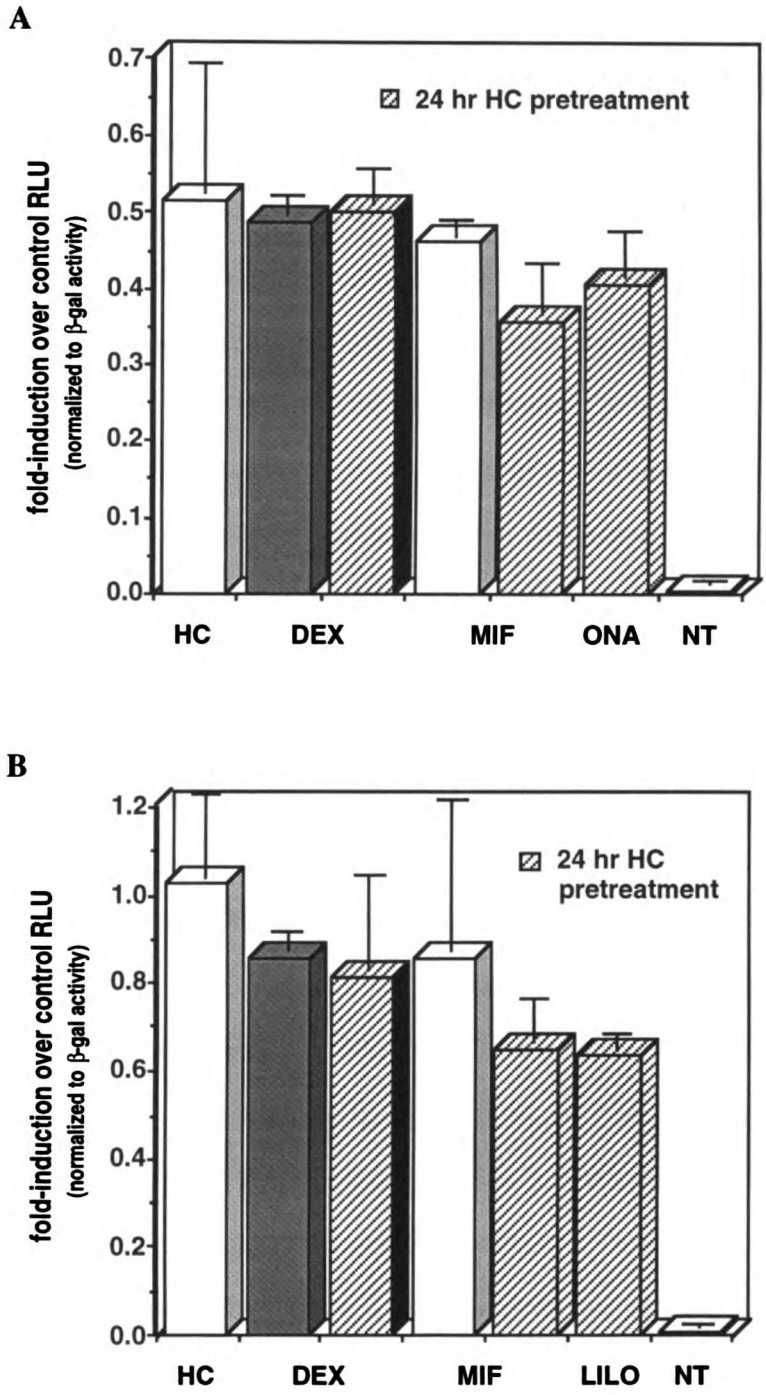


Figure 5.5 Effects of hydrocortisone pretreatment (24 hr) on subsequent effects of DEX, MIF, onapristone (ONA) and lilopristone (LILO) in **(A)** HepG2 and **(B)** Huh-7 cells (second treatments were 24 hr). HC, DEX and MIF treatments for 48 hr (nonhatched bars) were also without effect. NT, nontransfected wells.

5.4.3 Cotransfection of expression vectors for rat GR and its DNA-binding domain

At this point HepG2 and Huh-7 appeared entirely unsuitable for the study of CYP3A4 regulation. In both cell types known inducers such as DEX, PB and RIF failed to transcriptionally activate luciferase expression from the 1.2 kb CYP3A4 regulatory region. It is entirely possible that the relevant, important elements lay further upstream. However, also likely is the absence of important regulatory proteins (e.g., receptors or transcription factors) necessary for an induction response. Schuetz et al. [165] recently reported that DEX mediated transcriptional activation of a construct consisting of 1.4 kb of the CYP3A5 regulatory region, a thymidine kinase promoter and a chloramphenicol acetyltransferase reporter gene required cotransfection of an expression vector for human GR. Because we hypothesized that MIF induces CYP3A4 through a similar mechanism to DEX, we decided to evaluate the effects of cotransfecting a GR expression vector in HepG2, with the aim of producing a reliable DEX response and then assessing the effects of the antiprogestins.

Cotransfection of 10-100 ng of the expression vector for rat GR, 6rRSVrGR, followed by 48 hr treatment of the cells with 10 μM DEX, clearly failed to facilitate luciferase induction from the construct (Fig. 5.6A). Moreover, there was a decrease in control luciferase activity with increasing amounts of the GR expression vector (Fig. 5.6A). Utilizing 200-1000 ng of the receptor expression vector resulted in further, drastic reductions in transfection efficiency and reporter activity with the increasing amounts of total DNA, prohibiting evaluation of DEX effects (data not shown). It was therefore decided to cotransfect the expression vector for the receptor DNA-binding domain (DBD) to maintain a constant total amount of DNA transfected, while increasing the proportion of vector for the full length receptor. Surprisingly, the 1:3 plasmid ratio (0.1 μg 6rRSVrGR and 0.3 μg 6rRSVrGR-DBD) produced a roughly 4.5-fold induction response with 48 hr DEX treatment, whereas a 1:1 plasmid ratio resulted in 4-fold induction (over decreased

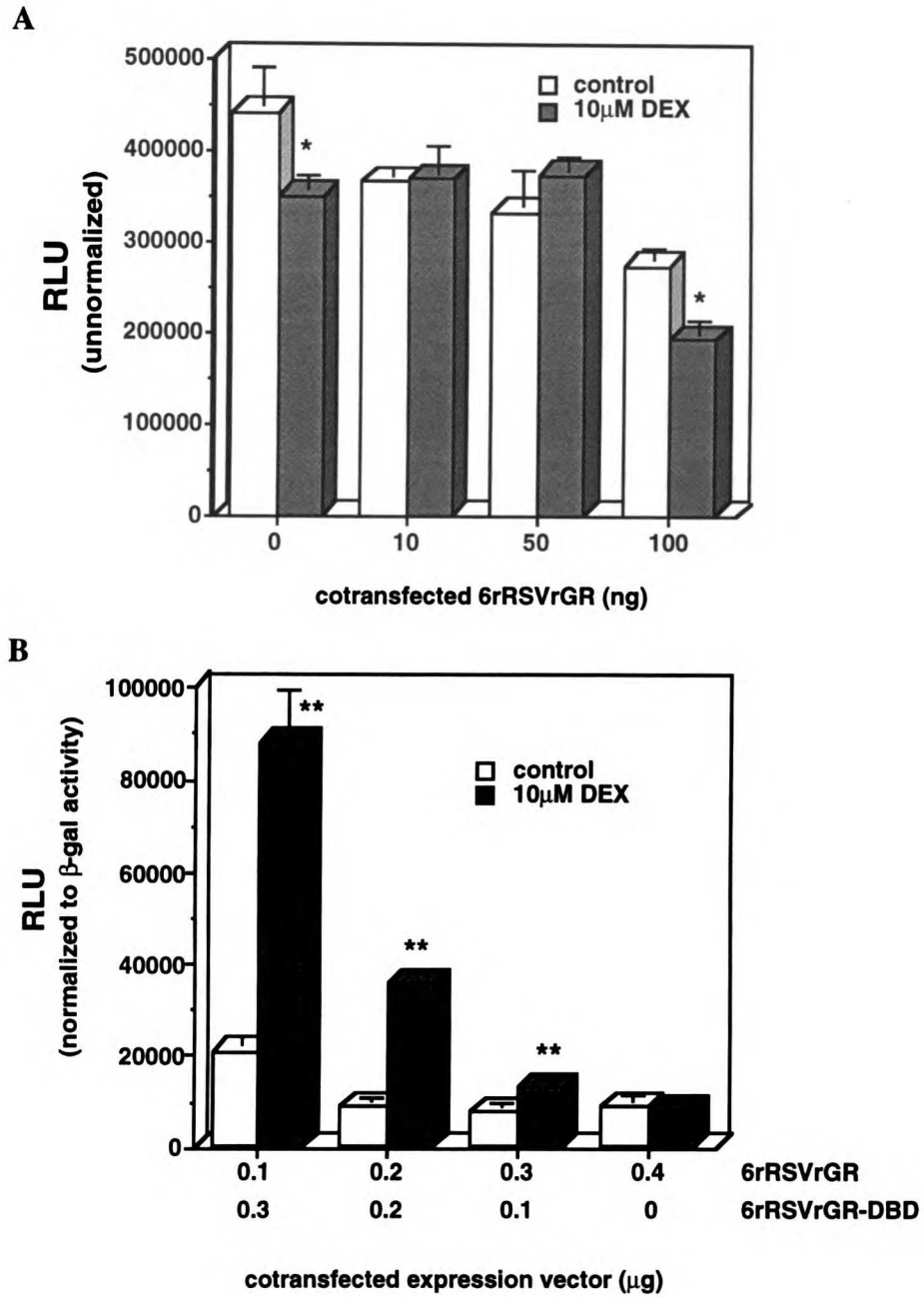


Figure 5.6 **(A)** Effects of cotransfecting 10-100 ng of an expression vector for the rat glucocorticoid receptor on dexamethasone mediated induction of the construct (48 hr treatment period). **(B)** Effects of cotransfecting expression vectors for both rGR and the receptor DNA-binding domain (DBD). *P<0.05, **P<0.001 relative to control.

control activity) (Fig. 5.6B). The 3:1 plasmid ratio produced modest induction (<2-fold) while the expression vector for the full-length receptor alone was without effect (Fig. 5.6B).

To confirm these unusual results, the experiment was repeated with the 0.1 μg:0.3 μg receptor to DBD vector ratio, as well as cotransfection of each construct individually. Cells were then treated with 10 μM DEX, MIF or ONA for 48 hr. The 1:3 ratio produced again, substantial (>6-fold) DEX mediated induction, a modest response to MIF (~1.5-fold induced) but no effect with onapristone (Fig. 5.7A). Cotransfection of 0.1 μg of the receptor expression vector alone resulted in a lower DEX response (~2.5-fold induction) and no effects with the antiprogestins, whereas the DBD vector itself was without effect for all three compounds (Fig. 5.7A). The 1:3 ratio was repeated again, along with a 1:7 ratio and a higher amount of the DBD vector alone. In this experiment 10 μM DEX, MIF and LILO treatments were performed for 48 hr. The DEX response was once more greater than 6-fold for the 1:3 plasmid ratio and roughly 4.5-fold for the 1:7 ratio (Fig. 5.7B). The MIF response was again modest and LILO treatment was non-inducing (Fig. 5.7B). Summarizing the results for all of the full-length receptor to DBD expression vector ratios tested, with subsequent DEX treatment, reveals a clear dose-response relationship (Fig. 5.8).

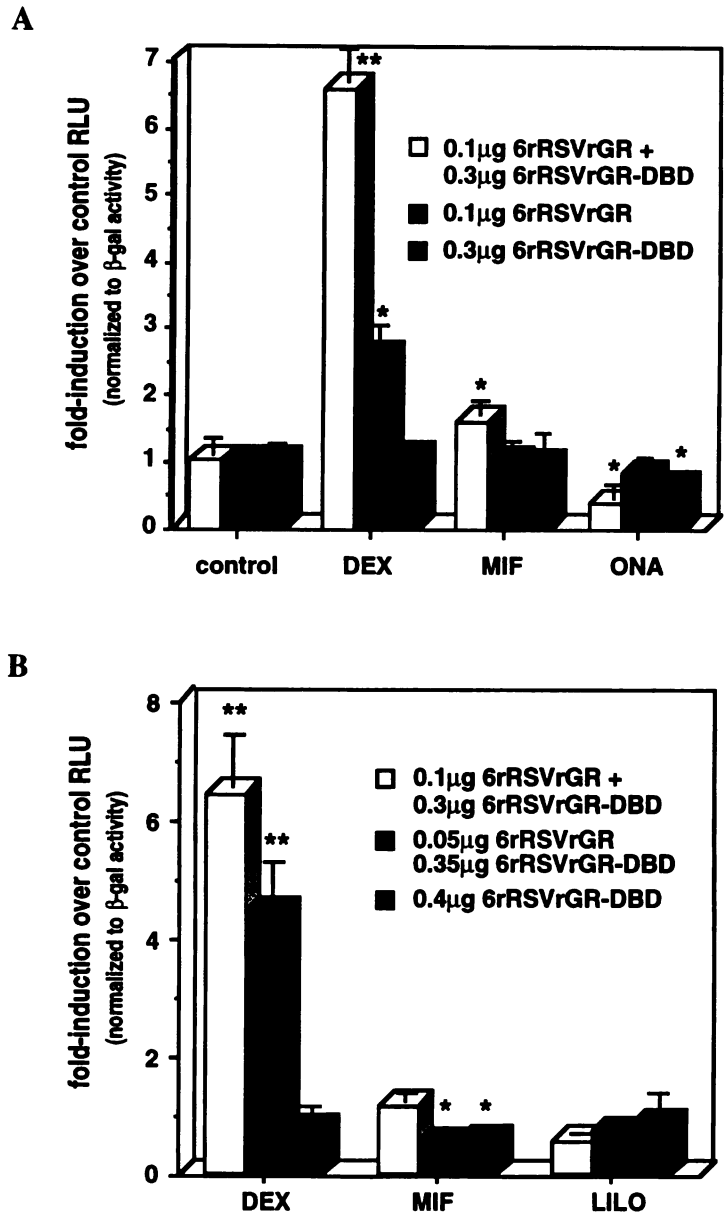


Figure 5.7 **(A)** Effects of cotransfecting the full-length receptor and DNA-binding domain (DBD) vectors in a 1:3 ratio, and individually, upon subsequent response to 10 μM DEX, MIF or ONA treatments. **(B)** Results of the same experiment with a 1:7 ratio, with a higher amount of DBD vector alone and in response to 10 μM LILO treatment. *P<0.05, **P<0.001.

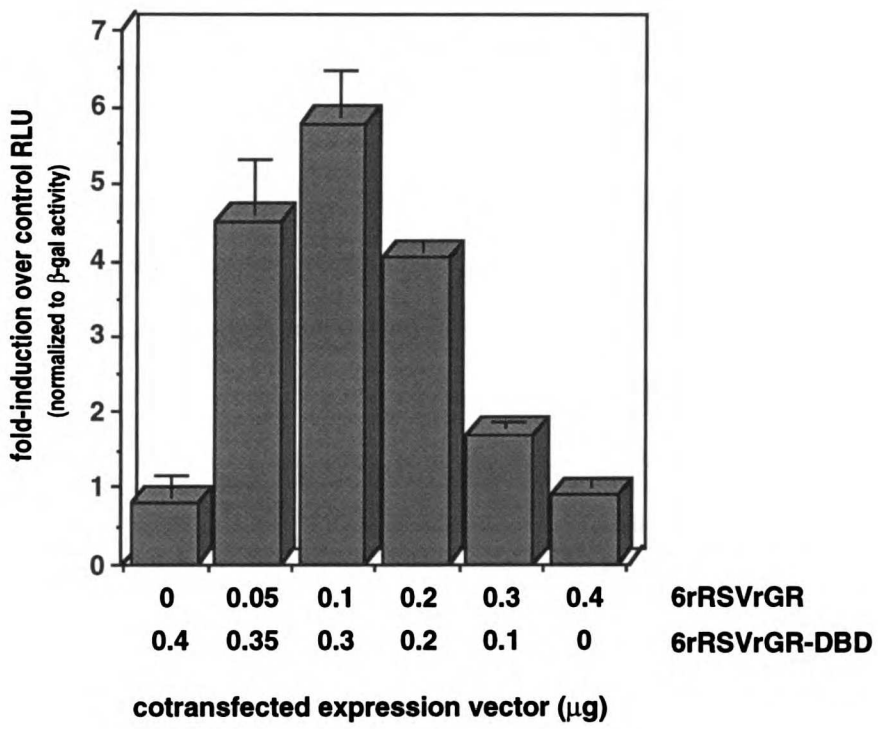


Figure 5.8 Dose-response relationship for induction of CYP3A4(-1105/+66)SV40-LUC by 10 μM DEX in HepG2 cotransfected with varying amounts of the rat GR and GR-DBD expression vectors (the 1:3 ratio is the mean±sd for the three separate experiments in which it was evaluated, N=9).

5.5 Discussion

These experiments demonstrate that HepG2 and Huh-7 are unsuitable for CYP3A4 regulation studies involving transient transfection of constructs incorporating the CYP3A4 5'-flanking region. Because of the aforementioned lack of a suitable or feasible model in which to study hormonal or antihormonal influences on CYP3A4 expression, we initiated these studies despite knowledge that HepG2 had been reported to lack constitutive expression of CYP3A4 and instead express CYP3A7 (the fetal-liver specific form) [166]. In that study, upon treatment of the cells with known CYP3A form inducers, 10 μM dexamethasone and rifampicin and 1 mM phenobarbital (as used in the present work), CYP3A7 but not other CYP3A forms was induced. We realized that this potentially indicated an inherent lack of transcription factors involved in CYP3A4 regulation in these cells, which may or may not be the same factors involved in an induction response.

However, as noted above, it was also reported that similar constructs incorporating 1.4 kb of the CYP3A5 regulatory region could be induced in HepG2 with cotransfection of an expression vector for the human glucocorticoid receptor [165]. This study demonstrated that two glucocorticoid response element "half-sites" spaced 160 bp apart governed the DEX response. Given that CYP3A5 is found in only ~20-30% of adult human livers and is thus polymorphically expressed [118], whereas CYP3A4 is not, it could be argued that the two genes are most likely regulated very differently. This is, at the very least, consistent with lack of restored CYP3A4 inducibility by cotransfected expression vector for the rat glucocorticoid receptor in our studies. Interestingly, this report of CYP3A5 induction contrasts with earlier reports of its noninducibility *in vivo* [118] and in a hepatocellular carcinoma cell line that constitutively expresses the protein [166]. It is most likely that findings related to CYP3A5 regulation should not be extrapolated to CYP3A4 regulatory mechanisms.

In contrast to several reports on HepG2, we were unable to find reports in which CYPs or their regulation had been investigated in Huh-7 cells. Our results indicate that potential lack of differentiation in this cell line precludes its use in CYP3A4 regulation studies. Analyses of CYP mRNA and immunoreactive protein expression in these cells might be of interest and would provide better assessment of their usefulness in general CYP studies, but such an undertaking may not be warranted based on our observations. Furthermore, CYPs are generally not expressed at significant levels in most cell lines.

The interesting finding that cotransfection of vectors encoding the full length glucocorticoid receptor and its DNA-binding domain in specific ratios results in a very strong and reproducible DEX response is difficult to interpret or rationalize. We requested a mutant receptor lacking in transcriptional activation potential (from the Yamamoto lab) to use as a negative control for anticipated effects with the full-length receptor. As noted above, the two vectors were cotransfected to maintain a constant total amount of transfected DNA (and thus to maintain a similar level of transfection). It was expected that the expressed DNA-binding domain would not interfere with the activity of the full-length receptor since it cannot bind ligand and should thus not compete with the receptor. The observed dose or ratio dependency for the DEX response with these two expression vectors suggests that it is not artifactual and, furthermore, may result from interaction between the two expressed proteins. For example, if HepG2 lack expression of one or more transcription factors crucial to glucocorticoid receptor mediated transcriptional activation, the DNA-binding domain may interact with dexamethasone-bound receptor dimers in a compensatory manner. Alternatively, potential interaction could affect receptor translocation or conformation, or be at the level of chromatin structure. However, it is also possible that the DNA-binding domain vector is affecting expression of the full length receptor from its expression vector. Our findings, although at present unexplainable, are consistent with a role of the glucocorticoid receptor in dexamethasone mediated induction of CYP3A4, in contrast to an earlier report of the contrary [92].

Shortly before commencing this work, Kocarek et al. [167] reported that mifepristone induced CYP3A forms at both the protein and mRNA levels in primary cultures of rat and rabbit hepatocytes. The antiprogestin was used to treat only one of five primary cultures of human hepatocytes used in the study, but in this culture CYP3A mRNA was increased nearly 3-fold (by 4 days treatment with the drug at 10 μM). Our initial hypothesis of mifepristone mediated transcriptional activation of CYP3A4 had thus been preliminarily confirmed. Our focus then was to assess the two newer agents and the endogenous hormonal effects should these cell lines prove suitable. Clearly it is difficult to draw conclusions from the observed results involving cotransfection of the expression vectors for the glucocorticoid receptor and its DNA-binding domain, but it appears that the two newer agents, lilopristone and onapristone, do not induce transcription from the CYP3A4 regulatory region, at least in a manner similar to that of dexamethasone. Mifepristone, in contrast, which from the above-cited work appears to induce CYP3A4 transcription, did produce modest induction of the construct with the optimal ratio found for the dexamethasone response. It therefore may be mediating this response also via its interactions with the glucocorticoid receptor. These conclusions are of course very preliminary, but are consistent with our hypothesis of mifepristone mediated induction of CYP3A4 and lack of induction by the newer agents, based on their diminished relative binding affinities to the glucocorticoid receptor.

Chapter 6 SUMMARY AND PERSPECTIVES

The metabolism of mifepristone and the two newer antiprogestational agents, lilopristone and onapristone, was characterized in human liver microsomes from three organ donors. For mifepristone, the first antiprogestin synthesized and used clinically, the metabolic profile *in vitro* reflected metabolite patterns observed *in vivo* in humans: rapid demethylation of the 11β-dimethylaminophenyl moiety, resulting in high levels of the mono-N-demethylated metabolite, followed by slower and less extensive accumulation of the didemethylated and 17α-propynyl-hydroxylated derivatives. For lilopristone and onapristone, which already possess hydroxyl groups on their 17-position substituents, only mono- and didemethylated metabolites were detectable. Relative to endogenous steroids, which are extensively hydroxylated by cytochromes P450 on their ring positions (particularly 6β), these antihormones are apparently not oxidized in an analogous manner. Divergent sites of metabolism between the hormones and antihormones could arise partly from different orientations of substrate binding in the active site of their metabolizing enzyme. The different orientations may be due to interactions of the bulky 11β substituent of the antiprogestins with residues in the substrate binding pocket and the absence of this functional group in the agonist molecules. Altered sites of metabolism are also the likely result of differences in reactivities of those functional groups which, as a result of binding orientation, are closest to the active O_2 species of the CYP.

For each of the three antiprogestins, evidence was obtained supporting a principal role of CYP3A4 in their oxidative metabolism. This was demonstrated initially for mifepristone. Chemical inhibitors selective for CYP3A enzymes substantially reduced initial rates of demethylation and hydroxylation of the compound, whereas inhibitors selective for other major CYPs involved in xenobiotic metabolism in humans were without effect. Polyclonal antibodies raised to CYP3A4 resulted in similar extents of inhibition of mifepristone oxidations. These data were further confirmed with correlation analyses, in which initial rates of oxidation of the compound correlated highly significantly with relative CYP3A immunoreactive protein levels and rates of CYP3A marker substrate metabolism in

a bank of human liver microsomes from 14 donors. Lilopristone and onapristone demethylations were similarly inhibited only by chemical inhibitors and antibodies specific to CYP3A enzymes. Thus, like numerous other steroidal molecules, both endogenous and synthetic, the antiprogestins are primarily metabolized by CYP3A4. Because several companies, such as Schering AG and Organon, continue to synthesize and develop new antiprogestin molecules, it would be of particular interest to study the metabolism of those antagonists lacking the 11β-dimethylaminophenyl moiety and thus the principal site of oxidation for the 3 compounds examined in the present work. Ring hydroxylation of these novel agents may occur in the absence of other, more favorable sites of oxidation.

The three antiprogestins were found to inactivate CYP3A4 in a time and NADPH-dependent manner. This was demonstrated utilizing the metabolism of midazolam, lilopristone and onapristone as markers of CYP3A4 catalytic activity. Spectrophotometric studies with human liver microsomes failed to detect a reactive nitroso species complexed to the CYP heme (demonstrating an absorbance maximum in the range of 445-455 nm). We suspect that this is due to insufficient levels of CYP3A forms in these microsomes. The identity of the reactive metabolite therefore remains unconfirmed, but the clinical implications of CYP3A4 inactivation are clear. Single-dose administration of one of these antiprogestins, for instance in pregnancy termination, would likely lead to inactivation of CYP3A4 in the intestine and liver. Depending upon the dose, complexation of the enzyme could be extensive or indeed complete. Concomitant or immediate (within 1-2 days) administration of another CYP3A4 substrate would result in its increased bioavailability (if taken orally) and decreased hepatic clearance (if it is a low hepatic extraction ratio drug), leading to a significant and perhaps drastic increase in the second drug's AUC and potential toxicity. This scenario would, of course, be more likely and problematic upon long-term or chronic administration of these antiprogestins for uses such as contraception or anticancer therapy. Newer antiprogestins that are similarly effective but possess alternate

11β groups should be free of this undesirable trait and would therefore be preferable for such indications.

Inactivation of CYP3A4 by the antiprogestins studied also raises questions about their ability to similarly inactivate cytochromes P450 involved in steroid biosynthesis. CYPs 11A1, 11B1, 11B2, 17 and 19 (the aromatase) catalyze steroid oxidations in adrenal and other steroid producing tissues, and thereby govern relative rates of steroid hormone production from cholesterol. It is possible that their active sites are less promiscuous than that of CYP3A4, but if they are capable of oxidizing these antiprogestins, and as a result being inactivated, long-term administration of the compounds could perturb plasma and tissue levels of endogenous steroid hormones. Studies utilizing recombinant forms of these CYPs would afford direct evaluation of this possibility. The antiprogestins may also cause changes in hormone levels due to perturbation of feedback regulatory mechanisms that result from antagonism at the receptor level.

The usefulness of the human hepatoma cell lines HepG2 and Huh-7 in CYP3A4 regulation studies was evaluated by transiently transfecting DNA constructs containing 1.2 kb of the *CYP3A4* 5'-flanking region linked to the luciferase reporter gene. Luciferase expression was not induced in either cell line by known activators of *CYP3A4* transcription or by the antiprogestins. Attempts to induce expression of or to cotransfect an expression vector for the glucocorticoid receptor, prior to treatments with the inducers, were without effect. Intriguingly, cotransfection of expression vectors for both the full length receptor and its DNA-binding domain resulted in a highly significant response to dexamethasone, a lowered but significant response to mifepristone, but no response to lilopristone or onapristone. This suggests a similar mechanism of CYP3A4 induction for dexamethasone and mifepristone via the glucocorticoid receptor, and is also consistent with lack of induction by lilopristone and onapristone due to their much lower relative binding affinities to the receptor. A better measure of the abilities of these antiprogestins to induce CYP3A4 might be expected from studies with human hepatocytes in primary culture, which have

been demonstrated repeatedly and by numerous investigators [166-171] to accurately model the *in vivo* response to CYP inducers. This model system is likely much more robust than the cell lines used in the present work, but was not feasible due to an inadequate supply of liver tissue. Replicating cells in culture often assume altered states of differentiation, which likely govern and/or change their response to known inducing agents or to test compounds (such as the antiprogestins) with the number of times they are passaged. Newer, as yet unpublished, reports of (non-liver derived) cell lines that constitutively express CYP3A4 suggest an alternative to human hepatocytes in primary culture for CYP3A4 regulation studies. An important caveat, however, is that culturing conditions and the transcription factors needed for basal CYP3A4 expression may not necessarily support CYP3A4 induction by all agents capable of doing so *in vivo*. This seems a distinct possibility since CYP3A4 regulation and induction may occur through numerous mechanisms (e.g., via interactions at different regulatory elements in the 5'-flanking region or through receptor and non-receptor mediated mechanisms).

The findings reported in this dissertation suggest that, for these and newer antiprogestins, several of their characteristics, in addition to their relative binding affinities to the progesterone and glucocorticoid receptors and to α_1-acid glycoprotein, could adversely affect their pharmacokinetic characteristics, toxicities and effective clinical use. Mifepristone, which we demonstrate here to be a CYP3A4 substrate and inactivator, but which also appears to induce the enzyme [167], was recently found not to display time-dependent changes in its pharmacokinetics upon prolonged administration of 8 mg per day (a dose that does not exhibit a zero-order phase resulting from saturable protein binding) [172]. Induction of the enzyme in the intestine and liver by mifepristone may have compensated for its inactivation. If, as we suspect, lilopristone and onapristone inactivate but do not induce the enzyme, their prolonged administration may result in time-dependent decreases in their hepatic clearances and in their accumulation in the body.

In the ongoing syntheses and characterization of newer antiprogestins, a compound designed or modified to be exquisitely selective for the progesterone receptor, that is sufficiently bioavailable orally and that does not inactivate or induce its metabolizing enzyme would obviously be the ideal therapeutic candidate. Such a compound is perhaps on the horizon and would allow the full potential of this novel, very promising class of therapeutic agents to be realized.

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