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Topical Review

The Biology of the P-Glycoproteins

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Introduction

Resistance of neoplastic cells to the cytotoxic action of multiple chemotherapeutic agents is a major obstacle in clinical cancer treatment. Some tumors are intrinsically resistant to multiple drugs, whereas other neoplasms acquire multidrug resistance (MDR) following exposure to cytotoxic agents. The molecular basis for MDR is starting to be unraveled. The discovery of p-glycoprotein, a 170 kD plasma membrane protein that functions as an ATP-driven chemotherapeutic drug efflux pump in cancer cells, was a major advance in the field of drug resistance. Biochemical investigations revealed that p-glycoprotein-mediated drug efflux could be inhibited by a number of agents. Clinical scientists were quick to initiate clinical trials combining chemotherapeutic agents with reversing agents to treat drug refractory neoplasms. Basic scientists, intrigued by the finding that p-glycoproteins are present in normal tissues, have sought to define a role for these proteins in normal cellular physiology.

The p-glycoproteins are actually a family of proteins. One group mediates MDR while the function of another group, which has no role in MDR, is unknown. Recent evidence suggests these p-glycoproteins may be involved in membrane phospholipid transport.

Progress in p-glycoprotein research has been rapid and the subject of several reviews [68, 77, 81, 82, 145, 146]. This review will focus on the biology and biochemistry of the p-glycoproteins.

Key words: Drug resistance — ATPases — Chemotherapy — Cancer — Membrane Transport — Xenobiotics

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The Multidrug Resistance Phenotype is Defined in Cell Culture

The development of MDR has been extensively studied in cancer cell lines. MDR clones of these cancer cell lines developed by sequential selection in increasing concentrations of lipophilic cytotoxic drugs were resistant, not only to the selecting agent, but also to a number of structurally and functionally unrelated drugs [17, 126]. Cross resistance was seen between the *Vinca* alkaloids, anthracyclines, epipodophyllotoxins, actinomycin D and taxol (Table 1). Drug resistance was associated with decreased intracellular drug accumulation which was maintained by energy-dependent, enhanced drug efflux. The MDR phenotype in these cells could be reversed by a structurally diverse group of drugs (Table 2) including verapamil, cyclosporine, progesterone and trifluoperazine [75, 103, 184]. Concurrent administration of these chemosensitizers with chemotherapeutic drugs restored drug sensitivity in MDR cells, but had no effect on drug sensitivity in parental cell lines. Chemosensitization was associated with enhanced intracellular drug accumulation and decreased drug efflux.

MDR cell lines were found to overexpress a 170 kD plasma membrane glycoprotein [98, 143]. The level of expression of this protein, called p-glycoprotein, correlated with the degree of drug resistance and the magnitude of drug accumulation defects in MDR cells [17, 126]. Photoactive analogues of chemotherapy drugs, chemosensitizing agents and ATP were shown to bind to p-glycoprotein in MDR cell plasma membranes [18, 52, 123]. Kinetic analysis suggested that chemotherapeutic agents and MDR chemosensitizers were bound to a common site on p-glycoprotein. ATP binding was not blocked by chemotherapeutic drugs or chemosensitizers

Table 1. Chemotherapeutic agents associated with the multidrug resistance phenotype

Vinca alkaloids	Epipodophyllotoxins
Vincristine	Etoposide
Vinblastine	Teniposide
Colchicine	Taxol
Anthracyclines	Actinomycin D
Doxorubicin	
Daunorubicin	
Mitoxantrone	

Table 2. Chemosensitizing agents which reverse the multidrug resistance phenotype

Calcium channel blockers	Antibiotics
Verapamil	Cephalosporins
Diltiazem	Bafilomycin
Azidopine	Erythromycin
Dihydropyridines	Calmodulin inhibitors
Hormones and steroids	Trifluoperazine
Progesterone	Chlorpromazine
Tamoxifen	Lysosomotropic agents
Cortisol	Monensin
Hydrophobic peptides	Chloroquine
Cyclosporine	Miscellaneous
Valinomycin	Reserpine
Gramicidin	Yohimbine
Detergents	Quinidine
Tween 80	Staurosporine
Triton X-100	Dipyridamole
Nonidet P-40	FK 506

suggesting separate ATP and drug binding sites on p-glycoprotein.

P-Glycoproteins Are Members of the ABC Transporter Superfamily

P-glycoprotein genes have been cloned from mouse [61, 83, 85], human [73, 109, 142, 147, 189], hamster [127] and rat [60, 169] tissues. P-glycoprotein is encoded by a small multigene family. There are three gene products in rodents [60, 83, 85, 127] and two in man [145, 189]. The mouse genes are termed *mdr1* (or *mdr1b*), *mdr2* and *mdr3* (or *mdr1a*) [83, 85]. The human genes are MDR1 and MDR2 (or MDR3) [145, 189]. Transfection studies with full-length cDNAs divide the p-glycoprotein gene family into two groups. When transfected into drug-sensitive cells the Group I gene products, MDR1, *mdr1* and *mdr3*, confer the MDR phenotype [61, 83, 160, 185]. Transfection of Group II gene products, MDR2 or *mdr2*, however, does not confer drug resistance [61, 85, 157].

The full-length cDNA sequence for p-glycoprotein

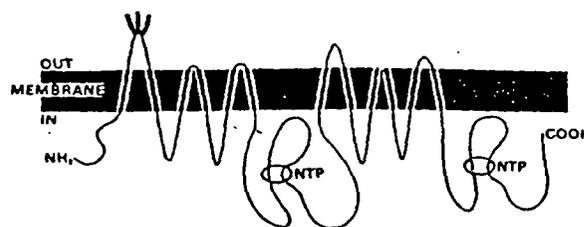


Fig. 1. Schematic diagram of p-glycoprotein structure. Reproduced, with permission, from *Advances in Pharmacology* 21, c1990, by Academic Press.

encodes a 1280 amino acid protein with significant homology between C and N terminal halves [40, 80, 83]. Hydrophobicity plots are most consistent with a topology which includes six transmembrane domains within each half of the protein (Fig. 1). The hydrophilic cytoplasmic portion of each half contains an approximately 200 amino acid consensus sequence common to all members of the ATP binding cassette (ABC) superfamily of membrane transport proteins [90]. This consensus region contains the ATP binding motifs known as the Walker A and B domains.

The majority of ABC proteins are involved in active membrane transport [90]. Approximately 50 prokaryotic members of the ABC superfamily have been identified [71]. These bacterial cell membrane proteins are involved in the ATP-dependent transport of a variety of substrates including proteins, peptides, amino acids, sugars, inorganic anions and polysaccharides. Homology of these prokaryotic proteins with p-glycoprotein resides primarily in the region surrounding the nucleotide binding domains. Several eukaryotic ABC transporters have been identified in yeast. The STE 6 gene product in *Saccharomyces cerevisiae*, which shares homology to p-glycoprotein in both the nucleotide binding region and within the transmembrane domains [105, 117], is responsible for the secretion of "a" mating pheromone across the yeast plasma membrane. The PDR5 and SNQ2 genes in *S. cerevisiae* [108, 128] and the *pmd 1 +* gene in *Schizosaccharomyces pombe* [161], which share less homology with p-glycoprotein than STE 6, are associated with fungal drug resistance. In *S. pombe* a gene associated with cadmium resistance, HMT 1, is involved in ATP-dependent transport of phytochelutins, small peptides equivalent to mammalian glutathione, across the yeast vacuolar membrane [130; D. Ortiz, *personal communication*). The *pfmdr1* gene in *Plasmodium falciparum* shares a high degree of homology to p-glycoprotein. The protein product of this gene has been localized to the trophozoite digestive vacuole where it may function in drug resistance [53, 54, 74, 102]. In *Leishmania sp.* several p-glycoprotein gene homologues have been identified in drug-resistant parasites [30, 49, 88, 131]. P-glycoprotein homologues have also been cloned from

Entamoeba histolytica [152], *Caenorhabditis elegans* [156] *Drosophila* [195] and marine sponges [107]. The demonstration of p-glycoprotein gene homologues across phylogenetic lines suggests that p-glycoprotein is an ancient protein associated with fundamental cellular functions.

Several mammalian homologues of p-glycoprotein have been identified. The TAP1 and TAP2 proteins present on the endoplasmic reticulum of T lymphocytes are involved in the ATP-dependent transport of cytosolic peptides derived from internalized antigens into the lumen of the endoplasmic reticulum [62, 165]. A hepatic peroxisomal membrane protein, PMP 70, has sequence homology to p-glycoprotein [100]. When this protein is mutated, a fatal cerebro-hepato-renal dysfunction known as Zellweger syndrome develops [76]. The cystic fibrosis transmembrane conductance regulator protein, (CFTR), which is an ATP-dependent, cAMP-regulated chloride channel, also shares homology with p-glycoprotein [144].

Although the 12 transmembrane domain model for p-glycoprotein topology is supported by antibody localization data [198], recent studies suggest an alternative topographical model. Evaluation of the murine *mdr1* or human MDR1 gene products translated in a cell free system or expressed in *Xenopus* oocytes revealed a glycosylation site in the second half of p-glycoprotein, as well as the previously reported sites in the first extracellular loop [173, 202]. This second glycosylation site links transmembrane domains 8 and 9 in the traditional model of p-glycoprotein topology suggesting that they are located extracellularly. The authors propose a model for p-glycoprotein topology with 10 transmembrane domains.

The functional unit of p-glycoprotein in the plasma membrane has not been established. Recent studies have isolated oligomeric complexes of p-glycoprotein from detergent extracts of human and hamster MDR cells [135]. These oligomers were derived from the noncovalent association of p-glycoprotein monomers and were able to bind ATP.

P-Glycoproteins Are Present in Normal Tissue

Examination of *mdr* mRNA expression [55, 73, 157, 187] and immunomorphometric localization of p-glycoprotein [28, 157, 176, 181] revealed the presence of p-glycoprotein in normal tissues. P-glycoprotein is found on the apical surfaces of many secretory epithelial cells. The human MDR1 gene product is found on the brush borders of the intestinal epithelial cells and renal proximal tubule cells [181]. It has also been localized to cells of the adrenal cortex [181], trophoblastic cells of the placenta [176], capillary endothelial cells in the brain and testis [51] and on the surface of lymphocytes [38]

and hematopoietic stem cells [39]. Small amounts of MDR1 are found on the hepatic biliary canalicular membrane and on the apical surface of the small biliary and pancreatic ductules [73, 181]. In mice, the *mdr1* gene product has a distribution similar to MDR1 in the adrenal gland, kidney, pregnant uterus and placenta [10, 55]. The murine *mdr3* gene product is found primarily in the intestine with small amounts in the brain and on the hepatic biliary canalicular membrane [27, 55]. The murine *mdr2* gene product is the predominant p-glycoprotein on the biliary canalicular membrane with small amounts found in the adrenals, heart, muscle and B lymphocytes [27, 55]. Human MDR2 mRNA has been localized to the liver and adrenals [43, 157].

P-Glycoproteins Are Membrane ATPases

P-glycoprotein ATPase activity was investigated in plasma membrane fractions and partially purified and reconstituted protein preparations from MDR cells. The initial purification of p-glycoprotein from MDR hamster cells did not preserve ATPase activity [143]. P-glycoprotein purified from MDR human leukemia cells had low ATPase activity (1.2 nmol/min/mg protein) [86, 87]. Four groups have subsequently reported p-glycoprotein ATPase activity in partially purified soluble plasma membrane fractions derived from human or hamster MDR cells. In these preparations, p-glycoprotein ATPase activity ranged from 0.37–3.4 $\mu\text{mol}/\text{min}/\text{mg}$ protein [4, 9, 65, 162]. In the most highly purified (90% pure) preparation, hamster p-glycoprotein [162] had an ATPase activity of 0.321 $\mu\text{mol}/\text{min}/\text{mg}$ protein. P-glycoprotein ATPase activity has also been associated with plasma membranes from Sf9 cells transfected with human MDR1 cDNA, (0.03 $\mu\text{mol}/\text{min}/\text{mg}$ protein) [153] and with a beta-galactosidase p-glycoprotein fusion protein expressed in and purified from fibroblasts (0.18 $\mu\text{mol}/\text{min}/\text{mg}$ protein) [166]. In all preparations, p-glycoprotein ATPase activity was magnesium dependent and substitution with calcium supported little, if any, activity. ATP was the preferred substrate although GTP and ITP were hydrolyzed to some extent. The K_m for ATP ranged from 0.5 to 1.4 mM. Inhibitors of other known membrane ATPases, such as sodium azide, oligomycin, ouabain and EGTA, had no effect on p-glycoprotein enzyme activity. Vanadate, which acts as a transitional state analogue for the phosphate release from the P-type ATPases, inhibited p-glycoprotein ATPase activity, but at concentrations [1–12 μM] greater than those required for inhibition of the P-type ATPases (50–500 nM). The mechanism of vanadate inhibition of p-glycoprotein is unknown. No phosphorylated intermediate has been demonstrated for p-glycoprotein and the aspartyl-phosphate site conserved in the P-type ATPases is not present in p-glycoprotein. A different site for covalent enzyme phosphate complex formation may be present.

Sulfhydryl reagents, such as *N*-ethylmaleimide and HgCl_2 , inhibited p-glycoprotein ATPase activity [4, 65, 162, 166] implying the presence of an important cysteine residue in the p-glycoprotein catalytic and/or nucleotide binding domains. *N*-ethylmaleimide inhibition of p-glycoprotein ATPase activity in hamster MDR plasma membranes was protected by ATP and was not reversible with dithiothreitol [5]. The important sulfhydryls were shown to be localized in both the C and N terminal halves of the molecule.

Substrate-stimulated P-glycoprotein ATPase activity was demonstrated in plasma membrane fractions prepared from transfected Sf9 insect cells [153] or MDR hamster cells [4]. In partially solubilized membrane fractions from MDR human or hamster cells, drug-stimulated activity could not be demonstrated until the preparations were reconstituted into proteoliposomes [9, 162, 164]. Apparently, p-glycoprotein requires stabilization of its transmembrane domains in a lipid environment for optimum ATPase activity [66]. ATPase activity of partially purified reconstituted human p-glycoprotein was stimulated three- to fourfold by doxorubicin, daunorubicin, vinblastine, actinomycin D and verapamil [9]. None of these drugs changed the affinity of the ATPase for ATP. Drug-stimulated ATPase activity was inhibited by vanadate. In proteoliposomes containing partially purified hamster p-glycoprotein, ATPase activity was stimulated 50% by verapamil, trifluoperazine, progesterone and colchicine, whereas daunorubicin and vinblastine paradoxically inhibited ATPase activity [164]. ATPase activity in a highly purified hamster p-glycoprotein preparation was stimulated tenfold by verapamil and fourfold by vinblastine with lesser stimulation by other MDR active agents [162]. In this preparation, verapamil decreased the apparent affinity of ATP for the transporter as evidenced by an increase in the K_m for ATP from 0.5 to 2.76 mM. Differences in the degree of basal and drug-stimulated ATPase activity in these preparations could reflect differences in the lipid composition of the proteoliposomes used for reconstitution, effects of detergent solubilization, contamination with other membrane ATPases or differences in ATPase activity between human and hamster p-glycoprotein.

Group I P-Glycoproteins Mediate Drug Transport

Several groups have attempted to characterize p-glycoprotein-mediated drug transport in intact cells, plasma membrane vesicles or reconstituted proteoliposomes. A note of caution is advised in evaluating p-glycoprotein transport studies since a number of factors can complicate the generation of accurate kinetic data in these experiments. Since p-glycoprotein substrates are hydrophobic, they tend to partition into or bind to internal and external membranes. P-glycoprotein transport of hydro-

phobic substrates must be separated from membrane binding by demonstrating temperature dependence, inhibition by detergent permeation and/or sensitivity to osmotic collapse of the vesicular space. ATP-dependent transport of hydrophobic substrates must be differentiated from the diffusion of these compounds through membranes. Kinetic data must be calculated using initial rates of drug uptake and not steady-state levels of accumulation. At high concentrations, hydrophobic substrates tend to self-associate and complicate the generation of saturation curves necessary to make these calculations [58].

Plasma membrane vesicles from MDR human [95] and hamster [64] cells have been used to demonstrate ATP-dependent transport of chemotherapeutic drugs. In vesicles from human MDR cells, accumulation of vinblastine was osmotically sensitive and temperature and ATP dependent [95]. The K_m for ATP was 38 μM . Non-hydrolyzable ATP analogues did not support transport. Vanadate, verapamil and MDR active chemotherapeutic agents inhibited vinblastine accumulation. Colchicine and vinblastine accumulation in hamster MDR plasma membrane vesicles was saturable, osmotically sensitive, ATP dependent and disrupted by membrane detergent permeation [64]. Transport was inhibited by MDR active agents, vanadate and *N*-ethylmaleimide. Drug transport has been demonstrated in partially purified hamster p-glycoprotein reconstituted into proteoliposomes [164]. Uptake of colchicine into proteoliposomes was ATP and temperature dependent, osmotically sensitive and saturable. Transport was inhibited by daunorubicin, vinblastine and verapamil.

Apical membrane vesicles from normal rat tissues show p-glycoprotein-mediated drug transport. ATP-dependent daunorubicin and vinblastine transport was reported in inside-out rat biliary canalicular membranes vesicles (CMV) [101, 171]. Transport was osmotically sensitive and saturable with a K_m of 49 μM for daunorubicin and 26 μM for vinblastine. The K_m for ATP was 80 μM . GTP stimulated daunorubicin transport slightly, but nonhydrolyzable ATP analogues did not. Transport was sensitive to vanadate, but was unaffected by ouabain, sodium azide and *N*-ethylmaleimide. Other p-glycoprotein substrates inhibited daunorubicin transport. Rat intestinal brush border membrane vesicles also had ATP-dependent p-glycoprotein-mediated daunorubicin transport [167].

The kinetics of daunorubicin transport has been studied in intact MDR cells [175]. Saturable p-glycoprotein-mediated accumulation of daunorubicin with a K_m of approximately 1.5 μM was demonstrated. In these studies, substrate self-aggregation was monitored by fluorescent microscopy and values for the passive permeation of daunorubicin were determined so that diffusion, as well as p-glycoprotein-mediated pump activity, could be considered. In cell lines with low levels of p-glycoprotein,

the passive efflux rate of daunorubicin made a significant contribution to the total daunorubicin efflux rate. Only in cell lines with high levels of expression of p-glycoprotein did the contribution from passive efflux become negligible. Maximal transport velocities for daunorubicin correlated with the amount of p-glycoprotein in the cell lines.

P-glycoprotein-mediated drug transport has been studied in cell lines which form polarized epithelial surfaces when grown on porous supports. Net basal to apical transepithelial transport of vinblastine was demonstrated in the Madin Darby canine kidney cell line and in the human intestinal adenocarcinoma cell lines, CaCo-2, T₈₄ and HCT-8 [94, 97]. P-glycoprotein was immunolocalized to the apical surface of these cells. Transepithelial flux of vinblastine in these cells was inhibited by other MDR active agents and by monoclonal antibodies to p-glycoprotein.

The murine *mdr* genes have been expressed in secretory vesicle membranes in *S. cerevisiae* sec 6-4 mutants [148]. In these mutants, a temperature-sensitive defect in the final step of the vesicular secretory pathway allows the accumulation of large amounts of secretory vesicles containing newly synthesized plasma membrane proteins when the yeast are grown at the permissive temperature. Yeast sec 6-4 mutants transfected with either the murine *mdr1* or *mdr3* gene exhibited verapamil-sensitive vinblastine and colchicine accumulation [148].

Is There an Endogenous Substrate for Group I P-Glycoproteins?

Group I p-glycoproteins can function as ATP-dependent drug transport pumps in cancer cell lines and in some normal tissues. Although it is unlikely that chemotherapy drug transport is the primary function for p-glycoprotein in normal tissue, the search for an endogenous substrate for the protein has been elusive. Its polarized distribution on secretory epithelia and role in drug efflux suggest that p-glycoprotein may function in excretion of naturally occurring toxins or commonly encountered xenobiotics.

Recently, *mdr3* knockout mice were shown to have an increased sensitivity to the toxic effects of vinblastine [158]. When the same dose of vinblastine was given to normal mice and *mdr3* knockout mice, the knockout mice had higher vinblastine concentrations in many tissues at several time points after drug administration. The knockout mice also had a striking sensitivity to the neurotoxic effects of the hydrophobic antiparasitic compound, ivermectin. Large increases in the brain concentration of this drug were documented in the knockout mice most likely due to lack of p-glycoprotein-mediated drug transport across the cerebral endothelial cells of the blood-brain barrier.

Known substrates for p-glycoprotein represent a structurally and functionally diverse group of compounds, many of which are natural products of fungal, bacterial or plant origin [75]. The structures of some typical MDR substrates are shown in Fig. 2. Three-dimensional analysis of over 120 compounds known to interact with p-glycoprotein suggests that a p-glycoprotein substrate is a cationic, hydrophobic molecule with at least two planar rings and a molecular weight of 400–1500 [12, 132]. A number of naturally occurring compounds that share these characteristics have been tested in rat CMV for their ability to inhibit daunorubicin transport. To date, neither sterols, reduced or oxidized glutathione, epinephrine, various amines, sphingosine or prostaglandins E1, B1 and E2 have proven to be substrates [12].

Several observations suggest an interaction between progesterone and p-glycoprotein. Progesterone photoaffinity labels p-glycoprotein, inhibits the binding of chemotherapy drugs to p-glycoprotein and reverses the MDR phenotype in MDR cell lines [136]. Progesterone is not a substrate for the transporter, since steady-state levels of progesterone accumulation were the same in resistant and sensitive cells and no enhanced efflux of progesterone was demonstrated in MDR cell lines [197]. Levels of the *mdr1* gene product increase dramatically on the luminal surface of the secretory epithelium of the mouse uterus under the influence of progesterone [10, 11]. Progesterone has also been shown to regulate the activity of the murine *mdr1* promoter [133].

Since high levels of p-glycoprotein are found in the adrenal cortex where extensive steroidogenesis occurs, the role of glucocorticoids as endogenous substrates for p-glycoprotein has been explored. MDR cell lines show reduced accumulation and increased ATP-dependent efflux of cortisol [190]. Exposure to dexamethasone increased *mdr3* and *mdr1* mRNA expression in murine hepatoma cells [203]. In mouse Y1 adrenocortical carcinoma cells, increased steroidogenesis induced by adrenocortical hormone was accompanied by increased p-glycoprotein mRNA levels [3]. P-glycoprotein reversing agents disrupted steroid secretion in Y1 cells [44]. A null mutation in one allele of the murine *mdr1* gene in Y1 cells inhibited steroid secretion [3].

When transfected with a human adrenal MDR1 cDNA, a porcine renal proximal tubule cell line, LLC-PK1, which forms a polarized epithelial surface when grown on a porous support, had verapamil-sensitive transepithelial flux of vinblastine, cortisol, aldosterone and dexamethasone, but not progesterone [186]. In these cells, cortisol and dexamethasone did not inhibit azidopine photoaffinity labeling of p-glycoprotein whereas progesterone did. Slight differences in the structure among these steroids may determine whether a particular compound is transported by p-glycoprotein or simply competes for drug binding.

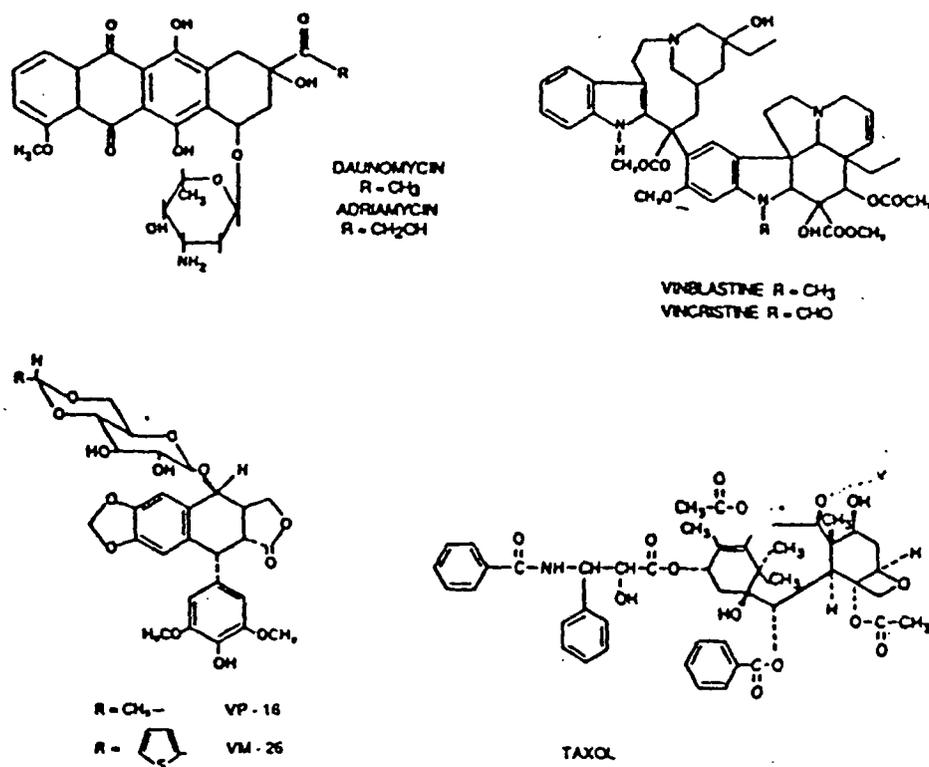


Fig. 2. Structure of some representative p-glycoprotein substrates. Reproduced, with permission, from the *Annual Review of Medicine* 42, c1991, by Annual Review.

P-glycoprotein is present on the capillary endothelium of the brain [51] where it may play a role in maintaining the blood-brain barrier. Lipophilic chemotherapy agents, such as those involved in the MDR phenotype, cannot enter the brain. The *mdr3* gene product is the predominant p-glycoprotein expressed in the murine brain [158]. When *mdr3* knockout mice and normal mice are given comparable doses of vinblastine, the knockout mice have profound increases in the brain concentration of this cytotoxic agent when compared to the brain concentration in normal mice [158]. Mouse brain capillary endothelial cell lines that stably expressed p-glycoprotein were established to study the role of p-glycoprotein in the blood brain barrier [177]. When grown on a porous filter, these cells demonstrated polarized p-glycoprotein expression and had verapamil-sensitive, unidirectional transepithelial transport of vinblastine. No transepithelial flux of fluorouracil, a non-MDR chemotherapy agent that is also excluded from the brain, could be demonstrated.

MDR hamster cells manifest verapamil- and vinblastine-sensitive, energy-dependent accumulation of morphine and binding of morphine to plasma membrane fractions [29]. Synthetic opiates including meperidine, pentazocine and methadone, increased vinblastine accumulation in MDR cells and inhibited photoaffinity labeling of plasma membranes with a doxorubicin analogue.

Homology of p-glycoprotein with several eukaryotic ABC transporters suggests a role for p-glycoprotein in

peptide transport. P-glycoprotein is highly homologous to the STE6 gene product in yeast which is responsible for the secretion of "a" mating factor, a hydrophobic dodecapeptide [105]. Expression of a cDNA for mouse *mdr1* [139] or human MDR1 [82] in yeast strains that lack the STE 6 gene product restored the ability to secrete the pheromone. P-glycoprotein shares a high degree of homology with the *hyl B* gene product in *Escherichia coli* that is responsible for the ATP-dependent secretion of the protein, hemolysin [71]. In *S. pombe*, a p-glycoprotein homologue transports phytochelatin, small gamma-glutamyl-cysteine-glycine tripeptides (D. Ortiz, *personal communication*). The TAP1 and TAP2 gene products, which transport cytosolic peptides into the endoplasmic reticulum, share homology with p-glycoprotein [62, 165]. P-glycoprotein expression and multidrug resistance were induced in tissue culture by exposure of hamster cells to the cytotoxic synthetic hydrophobic tripeptide, N-acetyl-leucyl-leucyl norleucine [163]. Cyclosporines, cyclic hydrophobic peptides, bind to and may be substrates for p-glycoprotein [150, 184]. Hamster MDR cells display verapamil-sensitive resistance to the toxic hydrophobic peptides, gramicidin D and valinomycin [113, 124, 149]. Since the liver normally functions to extract a variety of small peptides from the circulation, a number of synthetic and naturally occurring hydrophobic peptides, including insulin, cholecystokinin, neurokinin A and RNase-S-peptide, were tested for their ability to inhibit ATP-dependent

daunorubicin transport in rat CMV. Inhibition was not observed at peptide concentrations as high as 200 nM [12].

Group I P-Glycoproteins Interact with a Diverse Group of Lipophilic Compounds

How does p-glycoprotein interact with such a large variety of substrates? Substrate specificity has been addressed by examining the effect of sequence differences among cloned p-glycoprotein genes on drug resistance phenotypes and drug binding. Although the murine *mdr3* and *mdr1* gene are highly homologous (92% identity), when transfected into cells they resulted in different MDR phenotypes [61]. Cell clones transfected with the *mdr1* gene were less resistant to doxorubicin, colchicine and mitoxantrone than were those transfected with the *mdr3* gene. Cell clones with *mdr3* were more resistant to actinomycin D than were *mdr1* clones. Both the *mdr1* and *mdr3* genes are homologous to the *mdr2* gene (74% identity), yet the *mdr2* gene product is not associated with the MDR phenotype [85]. Examination of a series of chimeras, constructed throughout the whole length of *mdr1* with segments of *mdr2*, revealed that only the highly homologous ATP binding sites and the highly divergent linker regions were interchangeable enough to produce a functional molecule [28]. Similar results have been demonstrated with human MDR1 and MDR2 [82]. Another MDR1/MDR2 chimera, which had an 89 amino acid region of MDR2 including the first cytoplasmic loop and parts of the third and fourth TM domains substituted into MDR1, did not confer MDR, but when four amino acids in the cytoplasmic loop were changed back to those found in MDR1, biological function was restored [56]. This suggests that the first cytoplasmic loop is important for p-glycoprotein function.

Investigation of several point mutations or deletions in p-glycoprotein suggests that the predicted transmembrane domains play an important role in determining substrate binding and transport activity. A naturally occurring mutation of glycine to valine at position 185 in transmembrane domain 3 of human MDR1 changed the specificity of the transporter so that colchicine and etoposide resistance were enhanced while resistance to vinblastine, vincristine and actinomycin D was decreased [48, 104, 150]. Despite this resistance pattern, the mutant protein showed increased binding of vinblastine and decreased binding of colchicine. This discrepancy between the observed phenotype and drug binding suggested that the glycine to valine substitution did not affect initial drug binding, but instead altered the efficiency of dissociation of drugs from p-glycoprotein. Mutation of glycine 338 to alanine and alanine 339 to proline in transmembrane domain 6 in hamster p-glycoprotein decreased resistance to several drugs while maintaining

normal resistance to actinomycin D [63]. When serine 941 in transmembrane domain 11 of murine *mdr1* was mutated to phenylalanine, cell clones transfected with the mutated gene acquired resistance to vinblastine while colchicine and doxorubicin resistance was decreased [84].

Progress has been made in identifying the drug binding site on p-glycoprotein by examining proteolytic fragments of azidopine or azidoprazosin photoaffinity-labeled human MDR1 and murine *mdr1* [24, 82]. Both the amino terminal and carboxy terminal halves were labeled. Photoaffinity binding has been localized to two regions: (1) transmembrane domains 5 and 6 or the cytoplasmic domain immediately following TM6 and (2) a region within the transmembrane domain 12 or the cytoplasmic region immediately following TM 12 [82]. This labeling pattern is consistent with the presence of two different drug binding sites or the presence of one binding site which is formed by two homologous halves of the molecule.

Point mutations in either of the two conserved glycine and lysine residues residing within the A fold of the Walker consensus sequence in the *mdr1* gene resulted in loss of biological function despite persistent binding of azido ATP [13]. This result agreed with vesicle studies in which ATP hydrolysis, and not simply binding, was necessary for p-glycoprotein function. Amino terminal human MDR1 β -galactosidase fusion proteins expressed in fibroblasts were capable of ATP hydrolysis [166]. Expression of cDNAs encoding the amino or carboxy terminal half molecules of human MDR1 in Sf9 cells revealed that each half molecule had basal ATPase activity [114]. Drug-stimulated ATPase activity, however, was not present until both half molecules were expressed together. In separate studies, deletions of either the carboxy- or amino terminal half of p-glycoprotein resulted in loss of transport [57]. These results suggest that coupling of ATPase activity to drug binding and transport requires cooperative interaction between the two halves of p-glycoprotein.

Post-Translational Modification May Regulate P-Glycoprotein

P-glycoprotein is post-translationally modified by phosphorylation and glycosylation. Glycosylation does not appear to be necessary for protein function although it may play a role in the trafficking or stability of the protein [16, 110, 156].

Phosphorylation may have a role in regulating p-glycoprotein function. P-glycoprotein is phosphorylated by protein kinase C (PKC) [33, 34], protein kinase P [172] and protein kinase A (PKA) [118]. PKC inhibitors reduced the phosphorylation of p-glycoprotein and reversed the drug accumulation defect in MDR cells [32,

72, 115]. Cotransfection of a PKC-deficient breast carcinoma cell line with cDNAs for p-glycoprotein and PKC resulted in increased resistance to vinblastine and doxorubicin and increased phosphorylation of p-glycoprotein when compared to cells transfected with p-glycoprotein cDNA alone [199]. Basal levels of cAMP-dependent PKA were necessary to maintain p-glycoprotein mRNA levels in MDR cells [1, 44]. In vivo, p-glycoprotein was rapidly phosphorylated and dephosphorylated in MDR cells [115]. PKC phosphorylation sites in the human MDR1 gene product were mapped to multiple serine residues clustered in the linker region of the molecule [35]. Analysis of phosphorylation in the murine *mdr1* gene product mapped phosphorylation sites to the linker region, specifically on serine 669 for PKC and serine 681 for PKA [129]. The authors postulate that the *mdr* linker region is structurally equivalent to the R domain of the CFTR protein. Phosphorylation of the R domain by PKA regulates chloride permeability by CFTR. Although much smaller than the R domain (55 vs. 241 amino acids), the linker region in p-glycoprotein, like the R domain in CFTR, is encoded by a single exon, has several highly charged alternating basic and acidic amino acids and contains several PKA and PKC phosphorylation consensus sites [129].

Group I P-Glycoproteins Mediate Clinical Tumor Drug Resistance

There is strong evidence implicating p-glycoprotein as a clinically important mediator of multidrug resistance in human tumors. P-glycoprotein is frequently present in tumors derived from tissues normally expressing p-glycoprotein including hepatic, renal, intestinal and adrenal tumors [42, 73, 79, 96]. In general, these tumors tend to be intrinsically resistant to chemotherapy. Cancer cells derived from tissues that do not normally express p-glycoprotein acquire p-glycoprotein either as a result of malignant transformation [80] or after exposure to chemotherapeutic agents [31, 41, 59, 121]. In breast carcinoma [192], neuroblastoma [36], rhabdomyosarcoma [37], esophageal carcinoma [140], multiple myeloma [111], ovarian carcinoma [92], small cell lung cancer [92] and leukemia and lymphoma [134, 151, 154] the presence of p-glycoprotein in biopsy samples correlates with a poor response to chemotherapy.

Therapeutic strategies to circumvent p-glycoprotein action may influence the success of chemotherapeutic drug protocols. A large group of chemosensitizers interact with p-glycoprotein to reverse drug resistance in MDR cell lines [75, 103, 184]. This structurally diverse group of compounds (Table 2) includes calcium channel blockers, calmodulin antagonists, steroids and hormones, hydrophobic peptides, lysosomotropic agents and nonionic detergents. Some reversing agents including verapamil [200] cyclosporine [149, 184] and progester-

one [136, 197] appear to be competitive inhibitors that interact with p-glycoprotein at a common drug binding site. A note of caution, however, since kinetic analysis of these interactions is complicated by the hydrophobic nature of these compounds. Verapamil and cyclosporine may also be substrates for the transporter [149, 200]. Other agents, such as bile acids [116], are noncompetitive inhibitors interacting with p-glycoprotein at a site other than the drug binding site.

The growth of murine tumor xenografts derived from MDR cells can be inhibited by the administration of chemosensitizers along with chemotherapeutic agents [75, 77, 103]. Human clinical trials using p-glycoprotein chemosensitizers have been conducted [19, 168]. Some drug-refractory leukemias and myelomas responded to chemotherapy regimens when either verapamil or cyclosporine was added to the protocol [59, 112, 121]. Clinical trials with solid tumors, such as ovarian and colonic carcinoma, have met with limited success [193, 196]. Most of these clinical trials have been complicated by systemic toxicity. The maintenance of serum verapamil concentrations in the range associated with MDR reversal in vitro predictably led to dose-limiting cardiotoxicity [59, 121]. Patients given cyclosporine as a chemosensitizing agent developed hyperbilirubinemia [192, 196], consistent with this peptide's ability to inhibit ATP-dependent bile acid and nonbile acid organic anion transport in hepatocytes [99]. Clinical trials with trifluoperazine were limited by extrapyramidal side effects [120]. Progesterone, quinidine and tamoxifen have also been evaluated as chemosensitizing agents in clinical trials [19, 50, 168].

Expression of P-Glycoproteins Is Modulated by Environmental Stimuli

The regulation of p-glycoprotein gene expression in normal and neoplastic tissues is an area of active investigation. The liver serves as a good model system to study p-glycoprotein regulation since normal hepatic p-glycoprotein levels can be influenced by a number of environmental stimuli and the liver normally expresses both the Group I and II p-glycoprotein gene products which enables concurrent investigation of the effect of various factors on the expression of both gene products.

The induction of p-glycoprotein expression has been studied in hepatocarcinogenesis. Increased p-glycoprotein expression has been detected in clinical biopsy samples from human hepatocellular carcinoma (HCC) [42, 96]. In mice, overexpression of *mdr3* occurred in four models of HCC [106, 178], chemical carcinogenesis induced by dimethylhydrazine and diethylnitrosamine in C57BL/6N mice, spontaneous carcinogenesis in C3H/HeN mice and in tumors arising in transgenic mice carrying either the human hepatitis B virus large envelope polypeptide or the SV40 large T antigen. The *mdr3* gene

was activated in the latter stages of carcinogenesis in these models. Increased *mdr1* mRNA levels were occasionally seen, but *mdr2* mRNA levels did not change. In experimental models of HCC in rats, increased p-glycoprotein mRNA levels were detected in neoplastic tissue [23, 69, 182]. P-glycoprotein expression appeared to be associated with tumor progression rather than with tumor initiation in these rodent models. Several experimental observations support such a hypothesis. The human MDR1 gene promoter is a target for the Ras oncogene and the p53 tumor suppressor gene [47], both of which are associated with tumor progression. P-glycoprotein is usually heterogeneously expressed in clinical tumor samples, but was found in high concentrations in the invasive edges and distant metastases in colon carcinoma samples [194].

Rat liver cells transfected with v-H-Ras or v-Raf have increased p-glycoprotein expression and increased resistance to doxorubicin and vinblastine [25]. These results suggest that cellular transformation is sufficient to induce increased p-glycoprotein and that selective pressure from cytotoxic drugs is not necessary.

Hepatocyte p-glycoprotein expression can be influenced by other environmental factors. Hepatic regeneration induced by partial hepatectomy in rats was accompanied by increased expression of hepatic *mdr1a* and *mdr1b* RNA [125, 182]. These increases were noted within 24 hr and correlated with the previously described pattern of DNA replication seen in regenerating liver. In contrast, *mdr2* RNA levels were increased at 48 hr post hepatectomy after the major wave of cell proliferation had already occurred [125]. These results suggest differential regulation of the two gene products. In mice, *mdr1a* and *mdr2* mRNA levels were also increased in regenerating liver [179]. Cholestasis in rats, induced by bile duct ligation or exposure to alpha-naphthylisothiocyanate, increased *mdr1a* and *mdr1b* mRNA levels [159]. In monkeys, alpha-naphthylisothiocyanate cholestasis induced both MDR1 and MDR2 mRNA expression in the liver [159]. Rodents fed various cytotoxic xenobiotic agents, including aflatoxin, acetyl-aminofluorene, dioxin, phenothiazide and isosafrole, have increased *mdr1a* and *mdr1b* RNA expression [26]. Acetyl-aminofluorene induction of *mdr1a* and *mdr1b* in rats was associated with a verapamil-sensitive increase in biliary excretion of vinblastine [159]. In rat primary hepatocyte cultures, increased p-glycoprotein expression occurred upon exposure of cells to cytotoxic agents [45]. Induction of hepatic p-glycoprotein expression secondary to a variety of environmental insults may represent part of a coordinated hepatic cytoprotective response which has already been shown to include induction of metallothioneins, cytochrome p450 enzymes and phase II drug conjugating enzymes such as glutathione-S-transferases and UDP glucuronyl transferases.

P-glycoprotein gene activation has been correlated

with cellular differentiation. Exposure of several human colonic carcinoma cell lines to the differentiating agents, sodium butyrate and dimethylsulfoxide, increased MDR1 mRNA levels [119]. These agents, however, did not alter p-glycoprotein gene expression in primary rat hepatocyte cultures [70]. Increased p-glycoprotein mRNA levels occurred in human colonic carcinoma cells exposed to verapamil and cyclosporine [89]. Concomitant with verapamil treatment were electron microscopic and biochemical changes consistent with increased differentiation. In human renal, colonic and gastric carcinomas, MDR1 expression has been histologically correlated with the degree of tumor differentiation [14, 15, 122].

Two heat-shock consensus elements have been identified in the major MDR1 promoter [47]. Exposure to heat shock or heavy metals increased MDR1 gene expression in human kidney cell lines, but not in liver, adrenal, cervical or colonic cell lines [46].

Little is known about the developmental regulation or expression of p-glycoprotein. MDR1 has been identified in human fetal tissues [191]. Recently, p-glycoprotein was detected in mouse oocytes and early cleavage embryos [67]. In embryo cultures, p-glycoprotein expression was associated with a verapamil reversible increase in daunorubicin accumulation. Treatment of pregnant female mice with verapamil increased the toxicity of doxorubicin toward the developing zygote. These results suggest p-glycoprotein may play a protective role in early embryogenesis.

Group II P-Glycoproteins May Function in Phospholipid Transport

The *mdr2* gene product is not capable of chemotherapeutic drug binding or transport [27,85], although its nuclear binding domain is capable of ATP hydrolysis [28]. In mice, this gene product is primarily localized on the hepatic biliary canalicular membrane [27]. Homozygous disruption of the *mdr2* gene in mice resulted in the absence of phospholipid in bile and the development of liver lesions [174]. By 6-12 weeks of age, *mdr2* knockout mice had scattered hepatocyte necrosis, proliferation of bile ducts and mild portal inflammation. Bile analysis showed an absence of phospholipid. Heterozygous mice had bile phospholipid values 50% of those of normal mice and developed no hepatic pathology. Biliary phospholipid is normally incorporated into mixed micelles with bile acids, the major component of bile. It was hypothesized that in the absence of phospholipid solubilization, bile acids, by virtue of their detergent action, damage the biliary canalicular membrane and the apical membranes of the biliary ductules, giving rise to the pathology seen in the *mdr2* knockout mice [174].

These studies suggest that the *mdr2* gene product may be involved in phospholipid transfer from hepato-

cytes to the bile. The protein could function by facilitating the entry or exit of phospholipids from the canalicular membrane. Alternatively, it might be involved in the movement of phospholipids across the canalicular membrane. The latter function could be accomplished if the *mdr2* gene product functioned as a flippase, translocating phospholipids from the inner to the outer leaflet of the canalicular membrane where they can then gain access to bile. The functional activity of a phosphatidylcholine flippase has been described in rat endoplasmic reticulum [21] and in CMV [20]. The relationship of the latter translocator to the *mdr2* gene product has not been investigated.

A Number of Phenomena Associated with P-Glycoprotein Suggest Different Models of Action

Higgins and Gottesman hypothesized that the multidrug transporter might actually function as a "hydrophobic vacuum cleaner" which removes drugs from the plasma membrane before they reach the cytoplasm [82, 91]. This model of transport, they claim, would explain some puzzling aspects of the MDR phenotype. First, the broad substrate specificity of the transporter might be accounted for. In their model, the primary determinant of substrate specificity is the ability of a drug to interact with the lipid bilayer and secondarily to interact with the binding site of the transporter. This fits with the finding that all p-glycoprotein substrates are lipophilic compounds and that the major determinant of the ability of a given substance to be transported by p-glycoprotein is its relative hydrophobicity [201]. Second, the discrepancies in the kinetics of p-glycoprotein transport might be explained. These kinetic aberrations include an inability to correlate initial rate of drug efflux with p-glycoprotein concentration in MDR cells, differences between the level of drug resistance and the magnitude of drug accumulation defects seen in MDR cells and differences between the affinity constants determined in vesicle systems with the actual concentrations of cytotoxic agents that the transporter is able to protect cells from. In the Gottesman and Higgins model, drugs first partition into the lipid bilayer and then interact with p-glycoprotein. The actual drug concentration seen by the transporter would not correspond to the concentration of drug used in the experiment, but would depend on the ability of the drug to partition into the lipid bilayer, as well as on the lipid composition of the membrane. Changes in membrane fluidity have been shown to alter drug transport in rat CMV [171].

Several observations support the Gottesman and Higgins model. Doxorubicin photoactivates a highly lipophilic iodinated naphthalene azide membrane probe in MDR cells, but not in their sensitive counterparts [138]. In MDR cells, photosensitization was associated with

exclusive localization of doxorubicin to the plasma membrane. MDR1-transfected fibroblasts extrude the hydrophobic acetoxy methyl ester derivatives of several fluorescent dyes [93]. The free acid forms of these dyes, rapidly formed by the action of cytoplasmic esterases, are not extruded by the transporter. The free acid forms fail to accumulate in MDR cells, suggesting that the intact dye may interact with p-glycoprotein before it ever enters the cytoplasm [93].

Chloride channel activity was demonstrated in MDR1-transfected cells [6, 7, 188]. Another ABC protein, CFTR, has already been shown to be a chloride channel. MDR1-associated chloride currents exhibited rectification and were activated by changes in cell volume [188], whereas CFTR is a linear, small conductance channel activated by PKA-dependent phosphorylation in response to elevated cAMP levels [144]. In situ hybridization experiments showed complementary patterns of MDR1 and CFTR gene expression in epithelial tissues [183] suggesting that the two genes may be coordinately regulated. Epithelial cells that expressed CFTR lacked MDR1 and vice versa. In some tissues, different cell subpopulations preferentially expressed one of the gene products. For example, intestinal crypt cells expressed CFTR, whereas the apical villus cells expressed MDR1.

The electrophysiological properties of MDR1-associated chloride channel activity are similar to the volume-regulated chloride channels present in many epithelial cells, suggesting that MDR1 chloride channel activity may have a physiologic role in cell volume control. Recent studies in MDR1-transfected human breast cancer cells, which have p-glycoprotein-like chloride channel activity, failed to show that these channels were involved in volume regulation in these cells [6].

Chloride channel activity and multidrug transport appeared to be separate functions of p-glycoprotein. Agents which inhibited chloride channel activity in MDR1-transfected fibroblasts had no effect on multidrug transport [78]. In fibroblasts transfected with a MDR1 gene containing mutations in the ATP binding site, drug resistance was abolished, but chloride channel activity was maintained [78]. Hyposmotic solutions activated chloride channels in MDR1-transfected human breast cancer cells, but did not disturb p-glycoprotein-mediated efflux of the fluorescent dye, rhodamine [7]. Recent studies have cast doubt on p-glycoprotein's role as a chloride channel [137]. These experiments failed to show a quantitative correlation between p-glycoprotein expression and the activity of volume-sensitive chloride channel activity in four epithelial cell lines.

ATP channel activity has been ascribed to p-glycoprotein [2]. Channel activity was spontaneous and not associated with changes in cell volume. The authors suggest that the driving force for drug extrusion via p-glycoprotein might be an electrochemical gradient of ATP rather than ATP hydrolysis.

Many MDR cell lines have elevated intracellular pH [22, 170, 180]. In a series of myeloma cell lines which were sequentially selected for different levels of MDR1 expression, a linear relationship between intracellular pH and the initial rate of chemotherapeutic drug efflux was demonstrated [141]. In contrast, the initial rate of drug efflux was not associated with the level of p-glycoprotein expression. The authors postulate that p-glycoprotein is involved in intracellular alkalinization and is only indirectly involved in drug transport possibly by mediating a pH-dependent alteration in either membrane drug partitioning in the membrane or intracellular/extracellular distribution of cationic drugs.

P-glycoprotein's role in altering intracellular pH in MDR cell lines has been challenged. Some studies have failed to document consistent changes in intracellular pH in MDR cells [8]. In hamster and human MDR cell lines, experimental manipulation of the intracellular pH failed to effect p-glycoprotein-mediated drug efflux [8]. In yeast secretory vesicles expressing *mdr3*, vinblastine accumulation was independent of proton movements and unaffected by the presence or absence of a steep proton gradient [148].

Conclusions

The initial discovery of p-glycoprotein in the plasma membrane of MDR cancer cell lines was followed quickly by the cloning of its gene. Sequence analysis of cloned cDNAs revealed that p-glycoprotein was a member of the ABC family of membrane transporters. Subsequent biochemical characterization demonstrated the binding of chemotherapeutic drugs and ATP to p-glycoprotein. P-glycoprotein-mediated drug transport and drug-stimulated ATPase activity were documented in plasma membrane vesicles and in proteoliposomes containing the partially purified protein. P-glycoprotein was shown to be phosphorylated and the effect of this modification on the protein's biological function was explored. P-glycoproteins were found in many normal tissues and their overexpression was documented in numerous cancers. An important role for p-glycoprotein in intrinsic and acquired drug resistance in clinical oncology was established. Despite all that has been learned about p-glycoprotein over the last few years, additional studies will be necessary to address the many questions that have been left unanswered. Determination of p-glycoprotein structure and membrane topology should help elucidate the nature of chemotherapeutic drug binding sites and the mechanism whereby drug movement is coupled to ATP hydrolysis. Complete purification and functional reconstitution of p-glycoprotein into defined lipid vesicles will permit further characterization of drug transport and ATPase activity and give us the means by which p-glycoprotein's apparent dual function as a trans-

porter and a channel can be clarified. Structural and functional studies on p-glycoprotein will also provide information needed to develop specified inhibitors that can be used clinically to overcome MDR in cancer patients. Further study of the mechanisms whereby p-glycoprotein expression is induced and regulated during malignant transformation is indicated. The development of biliary phospholipid deficiency in *mdr2* knockout mice and xenobiotic hypersensitivity in *mdr3* knockout mice have given us the first clues into the normal physiologic roles for the p-glycoproteins. The search for endogenous substrates for the p-glycoproteins will continue to be an area of active investigation.

Continued investigation of p-glycoprotein's functions should result in better understanding of an important class of prokaryotic and eukaryotic membrane transporters. The potential of exploiting the knowledge garnered from these studies in the treatment of neoplastic, parasitic and inherited and acquired liver disease may be greater than we can now imagine.

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Note Added in Proof: A recent study (Ruetz, S., Gros, P. 1994. *Cell* 77:1-20) utilizing yeast secretory vesicles which overexpress the *mdr2* gene product has conclusively demonstrated that this protein functions as an ATP-dependent phosphatidylcholine translocase.

Impact of Tamoxifen on the Pharmacokinetics and Endocrine Effects of the Aromatase Inhibitor Letrozole in Postmenopausal Women with Breast Cancer¹

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ABSTRACT

This study examined whether the addition of tamoxifen to the treatment regimen of patients with advanced breast cancer being treated with the aromatase inhibitor letrozole led to any pharmacokinetic or pharmacodynamic interaction. Twelve of 17 patients completed the core period of the trial in which 2.5 mg/day letrozole was administered alone for 6 weeks and in combination with 20 mg/day tamoxifen for the subsequent 6 weeks. Patients responding to treatment continued on the combination until progression of disease or any other reason for discontinuation. Plasma levels of letrozole were measured at the end of the 6-week periods of treatment with letrozole alone and the combination and once more between 4 and 8 months on combination therapy. No further measurements were done thereafter. Hormone levels were measured at 2-week intervals throughout the core period. Marked suppression of estradiol, estrone, and estrone sulfate occurred with letrozole treatment, and this was not significantly affected by the addition of tamoxifen. However, plasma levels of letrozole were reduced by a mean 37.6% during combination therapy ($P < 0.0001$), and this reduction persisted after 4–8 months of combination therapy. Letrozole is the first drug to be described in which this pharmacokinetic interaction occurs with tamoxifen. The mechanism is likely to be a consequence of an induction of letrozole-metabolizing enzymes by tamoxifen but was not further addressed in this study. It is possible that the antitumor efficacy of letrozole may be affected. Thus, sequential therapy may be preferable with these two

drugs. It is not known whether tamoxifen interacts with other members of this class of drugs or with other drugs in combination.

INTRODUCTION

Estrogen deprivation is the primary mechanism of action of hormonal therapies in breast cancer. There are two main ways in which this may be achieved. The most frequent approach is to use an antiestrogen such as tamoxifen to antagonize estrogens at the estrogen receptor. An alternative is to reduce the synthesis of estrogens. In postmenopausal women, this is achieved by inhibition of the aromatase enzyme system, which is responsible for the generation of E_1 and E_2 from androgenic precursors.

Letrozole (CGS 20267) is a highly potent and specific nonsteroidal inhibitor of the aromatase enzyme system (1). Previous studies have demonstrated that letrozole in single oral doses ranging from 0.1–2.5 mg/day produces significant decreases in circulating E_2 and E_1 concentrations (2). The efficacy of aromatase inhibition has been found to be greater than 98% at the 0.5 mg/day dose and >99% at the 2.5 mg/day dose (1). A pivotal Phase IIb/III comparative study of 0.5 mg/day letrozole versus 2.5 mg/day letrozole versus 160 mg/day megestrol acetate found that the higher dose of letrozole produced a significantly higher objective response rate (24%) compared with megestrol acetate (16%) or 0.5 mg/day letrozole (13%; Ref. 3). Time to progression and time to treatment failure were also better at the 2.5 mg/day dose of letrozole than with megestrol acetate or 0.5 mg/day letrozole. This higher dose of letrozole was also found to achieve greater efficacy than the 0.5 mg dose in another Phase IIb/III study (4): there was a significant dose effect on overall survival in favor of 2.5 mg letrozole compared with 0.5 mg letrozole. The improved tolerability of letrozole and other aromatase inhibitors compared with megestrol acetate and AG³ (3, 4) has now led to them being the first choice endocrine therapy after tamoxifen for most patients. Their efficacy and excellent tolerability have also led to their incorporation into large-scale first-line and adjuvant trials versus tamoxifen in early breast cancer.

The differences in the mechanism of action between aromatase inhibitors and tamoxifen and observations that some patients who are resistant to tamoxifen respond to an aromatase inhibitor (5, 6) have led to a series of clinical trials combining the two agents. Until now, these trials have all used AG as the aromatase inhibitor (6–8).

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³ The abbreviations used are: AG, aminoglutethimide; SHBG, sex hormone-binding globulin; LH, luteinizing hormone; FSH, follicle-stimulating hormone; AUC, ; CV, coefficient of variation; CI, confidence interval; E_1 , estrone; E_2 , 17 β -estradiol; E.S., estrone sulfate; CYP, cytochrome P-450.

It is generally accepted that the studies combining the aromatase inhibitor AG and tamoxifen have not led to major gains in patient benefit (6-8). A possible explanation of the apparent lack of additive benefit may be the enhancement of tamoxifen clearance, which has been found to occur in the presence of AG and results in plasma tamoxifen levels of about only 30% of those in patients treated with tamoxifen alone (9). Animal studies of a combination of aromatase inhibitor and tamoxifen are also mixed in their findings. Some indicate improved efficacy over single-agent treatment (10), whereas others show a marked benefit for the single agent (11). Thus consideration has been given to whether the combination of letrozole and tamoxifen might improve the status of patients with advanced breast cancer and their time to progression.

Because it was not known whether these two drugs would interact, a two-center, open-label, nonrandomized, within-patient comparison of the pharmacokinetics and endocrine interactions of letrozole and tamoxifen was conducted in postmenopausal women with advanced breast cancer who could benefit from treatment with aromatase inhibitors and/or tamoxifen. The primary objective was to investigate whether cotreatment of patients who had already been on letrozole treatment for 6 weeks with 6 weeks of tamoxifen influenced the pharmacokinetic profile of letrozole. The secondary objectives were as follows: (a) to assess the safety and tolerability of the treatment combination of letrozole and tamoxifen; (b) to evaluate the effects on serum hormone levels (E_1 , E_2 , E_1S , SHBG, LH, and FSH); and (c) to evaluate patients for response and time to disease progression.

A separate study has been conducted to investigate whether letrozole influenced the pharmacokinetic profile of tamoxifen (12).

PATIENTS AND METHODS

Sample Size

Previous trials with letrozole (data on file; Novartis) indicated that the within-patient CV of AUC should not exceed 18%. Based on this CV, a sample size of 12 patients with completed pharmacokinetic profiles was calculated, which would provide an approximately 80% probability that the 90% CI for the ratio of AUC(letrozole/tamoxifen) to AUC(letrozole) would be contained within the equivalence limits of 0.8 and 1.25 if the two treatments were identical, *i.e.*, if the addition of tamoxifen did not affect the AUC(letrozole).

Trial Population and Previous Treatments

The trial population consisted of postmenopausal patients with locally advanced or locoregional recurrent or metastatic breast cancer who were eligible for treatment with endocrine therapy. Patients had to have a WHO performance status of grade 0-2 and a life expectancy of at least 3 months and must have provided written informed consent to the specific protocol. All patients had documented evaluable or measurable disease with objective evidence of disease progression. Patients with estrogen receptor-negative status and patients with conditions that would prohibit proper follow-up were excluded from the trial. Previous treatment with any of the following agents was not allowed: (a) antiestrogen therapy within the last 6 months;

(b) aromatase inhibitors; (c) other hormonal agents (*e.g.*, medroxyprogesterone acetate, megestrol acetate) within the last 4 weeks; and (d) bisphosphonate therapy within 6 months of starting trial treatment, if bone metastases were the sole manifestation of advanced disease.

The core treatment ran for 12 weeks. Patients received 2.5 mg letrozole daily for the first 6 weeks and continued to receive a combination of letrozole (2.5 mg) and tamoxifen (20 mg) tablets once daily until progression or any other reason for withdrawal. Patients who failed to complete the core trial were replaced.

Blood Sampling and Analytical Methods

Samples were collected into EDTA-containing tubes on week 6 and week 12 for pharmacokinetics at time 0 (*i.e.*, just before taking the daily dose of letrozole) and at 1, 2, 4, 6, 8, and 24 h thereafter. Plasma was stored at -20°C until analysis. Plasma concentrations of letrozole were determined using a high-performance lipid chromatography method with CGP 47645 as the internal standard (13). The limit of quantification was 1.4 nmol/liter. Preliminary results from 10 patients indicated a pharmacokinetic interaction between the drugs, and for those patients still on trial, another PK sampling was done between 4 and 8 months on combination treatment to confirm whether the interaction persisted over time.

Steady-state AUC values during a dosing interval were calculated by the linear trapezoidal rule. All available samples were analyzed for letrozole concentration. AUC data of all patients with at least one complete pharmacokinetic profile were subjected to statistical analyses.

Blood for hormone analyses was collected into plain tubes before starting therapy and on weeks 2, 4, 6, 8, 10, and 12. Serum was stored at -20°C until analysis. Serum E_2 and E_1 levels were measured according to previously described methodology with assays having sensitivity limits of 3 and 10 pmol/liter, respectively (14, 15). E_1S was measured after an initial extraction of unconjugated (free) E_1 and hydrolysis of E_1S to free E_1 . E_1 was then measured after ether extraction and column chromatography on Lipidex 5000 using a solvent system of chloroform:hexane:methanol (50:50:1). [^3H] E_1S was added as a recovery control. The RIA was performed using the Diagnostic Services Laboratory kit (DSL-8700). The overall assay sensitivity was 10 pmol/liter. SHBG was measured using the Famos immunoradiometric assay kit, which has a detection limit of 0.5 nmol/liter and intra- and interassay CVs of 3.2% and 8.3%, respectively. LH and FSH were measured by Abbott AxSYM. All samples from the same patient were included in the same assay batch.

Clinical Tumor Evaluation

Antitumor activity was evaluated according to the UICC criteria (16) at baseline and every 3 months thereafter or when the patient discontinued treatment. Blastic and mixed bone lesions were evaluated for progression but not for response. Performance status (WHO scale) was recorded at baseline, 6 weeks, 3 months, and every 3 months thereafter, and adverse experiences were recorded at 2-week intervals for the first 12 weeks and every 3 months thereafter.

Statistical Methodology

Pharmacokinetics. A possible effect of tamoxifen coadministration on letrozole levels was evaluated using bioequivalence testing on log-transformed AUC values, *i.e.*, equivalence was accepted if the 90% CIs of the difference in logAUCs between two treatments were contained within the limits 0.8 and 1.25. A general linear model with a treatment effect and a patient effect was fitted to the log-transformed AUC values. First, an equivalence test was performed on the AUC values of the two combination periods (12 weeks *versus* >4 months of combination therapy). Next, provided the two combination periods were equivalent, equivalence was tested on the difference in logAUCs of letrozole alone *versus* the average of the two combination treatment periods.

Hormones. Estrogen suppression was described over time using summary statistics, geometric mean, and minimum and maximum on the log (natural base)-transformed values. For statistical purposes, when estrogen levels were below the detection limit of the assay, they were ascribed the value of the respective detection limit. In addition, SHBG, LH, and FSH were described over time using the mean, SD, quartiles, minimum, and maximum. The effect of cotreatment with tamoxifen on the hormone effects of letrozole was assessed with descriptive statistics.

RESULTS

A total of 17 patients were recruited to the trial. Two patients withdrew during monotherapy treatment and an additional 2 patients discontinued treatment at the 6 week visit. Thus, 13 patients continued into the combination treatment phase, and 12 patients completed at least 6 weeks of combination therapy. All patients were postmenopausal and had receptor-positive tumors. Their median age was 64.1 years (range, 44–88 years), and 94.1% of patients had a WHO status of 0–1.

Antitumor Efficacy. After 3 months of core treatment (6 weeks of letrozole monotherapy followed by 6 weeks of the combination treatment), 3 of 17 patients had a partial response, 9 patients remained stable, 3 patients progressed, and 2 patients were not assessable. Six months after starting study, 2 additional patients [5 of 17 patients in total; 29.4% (95% CI, 10.3–56.0%)] had achieved partial response.

Adverse Experiences. During the 6 weeks of monotherapy, 2 of 17 patients reported at least one serious adverse event, and 1 patient discontinued treatment due to an adverse event. The discontinued patient experienced a non-drug-related hematemesis after a single dose of study medication, whereas the other patient experienced a non-drug-related erythematous rash after 13 days of trial treatment. During the rest of the core trial and the follow-up period of the combination, one patient suffered from continuous hypochondrial pain that was reported as a non-drug-related serious adverse event. Another patient was discontinued on combination treatment as a result of depression and headache, which resolved after combination treatment was stopped. Adverse events reported during either phase were mainly mild to moderate in severity. The most commonly reported individual adverse events during both treatment phases, irrespective of trial drug relationship, were depression, fatigue, nausea, hot flushes, and abdominal pain. Weight increases

Table 1 Study drug-related adverse events (listed by body system) that were reported in $\geq 10\%$ of patients in either treatment period

Body system	Letrozole (n = 17) n (%)	Letrozole plus tamoxifen (n = 13) n (%)
Total patients with adverse events	13 (76.5)	13 (100.0)
Body as a whole	3 (17.6)	5 (38.5)
Fatigue	2 (11.8)	3 (23.1)
Weight increase	1 (5.9)	4 (30.8)
Digestive system	5 (29.4)	3 (23.1)
Dyspepsia	2 (11.8)	1 (7.7)
Nervous system	3 (17.6)	5 (38.5)
Depression	2 (11.8)	2 (15.4)
Skin and appendages	7 (41.2)	7 (53.8)
Hot flushes	3 (17.6)	3 (23.1)
Rash	1 (5.9)	2 (15.4)
Special senses	2 (11.8)	2 (15.4)
Urogenital and reproductive system	1 (5.9)	4 (30.8)
Vaginal spotting	1 (5.9)	2 (15.4)
Leukorrhea	0	2 (15.4)

(5.9% *versus* 30.8%) and coughing (5.9% *versus* 30.8%) were reported by more patients during combined therapy than during monotherapy. With these exceptions, and taking into account the small patient numbers, the pattern of adverse events and their frequency of reporting were similar during both treatment phases. It should also be noted that the combination treatment period extended over a longer time period, increasing the likelihood that more events would occur in the combination phase than in the limited 6-week time period of monotherapy.

The study drug-related adverse events are listed by body system for those reported in $\geq 10\%$ of patients in either treatment period in Table 1. The most commonly reported study drug-related adverse events were hot flushes, fatigue, and weight gain. Weight gain was most discrepant in both treatment phases and was observed in 4 of 13 patients during combined treatment and 1 of 17 patients during monotherapy. All of the most commonly reported ($\geq 10\%$) study drug-related adverse reactions were known to be associated with letrozole or tamoxifen. Coadministration of the two drugs did not result in an increased incidence of any event, with the possible exception of weight gain. However, even in this case, the numbers are too low for definitive comment. At baseline, all patients had a WHO performance status grade between 0 and 2, with the majority of patients having a WHO performance status of grade 1. This situation was essentially unchanged through the whole 12 weeks of the study, although one patient had deteriorated to grade 3 at 12 weeks. There were no changes of concern in the hematological and blood chemistry analyses during either of the study periods.

Pharmacokinetics. In all but one patient, letrozole plasma concentrations were lower when letrozole was given together with tamoxifen (Table 2). The levels after 4–8 months of combination therapy were, in general, similar to those after 6 weeks of combination treatment. Fig. 1 displays the mean letrozole plasma levels for the three treatment periods. The mean AUCs are shown in Fig. 2. The AUC values between 4 and 8 months on combination therapy were not significantly different from those after 6 weeks of combination treatment (90% CI,

Table 2 Letrozole AUC values (h.nmol/liter) for individual patients after 6 weeks monotherapy (letrozole, LET), and 6 weeks and >4 months combination therapy (letrozole + tamoxifen, LET + TAM)

	LET	LET + TAM (6 weeks)	LET + TAM (>4 months)
Patient 1	7570	4600	4980
Patient 2	4360	4590	4770
Patient 5	7880	6430	"
Patient 7	6310	4370	4110
Patient 8	14700	"	"
Patient 9	16400	6660	6500
Patient 10	8310	5210	6420
Patient 11	5840	3380	3048
Patient 12	11300	9840	9190
Patient 13	13300	5470	4040
Patient 15	11600	5670	5580
Patient 16	9570	6040	6590
Patient 17	10100	6630	7270
Patient 18	4090	"	"
Mean	9381	5741	5682
SD	3761	1636	1741
n	14	12	11

* Patient withdrew from the study before the profile being taken.

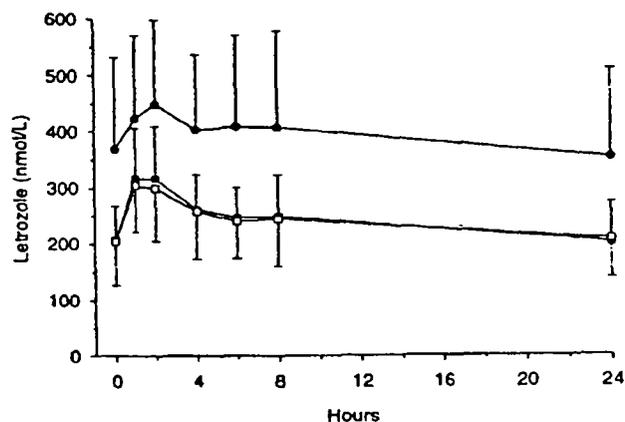


Fig. 1 Pharmacokinetic 24-h profiles of the mean \pm SD phase letrozole levels after 6 weeks of treatment with letrozole alone (\bullet), 6 weeks of treatment with letrozole plus tamoxifen (\blacksquare), and >4 months of treatment with letrozole plus tamoxifen (\square).

0.91–1.13). However, AUC values of letrozole treatment alone were significantly higher than those of the combination treatment: the ratio of AUC (letrozole alone) to AUC (combination) was 1.60 with a 90% CI of 1.46–1.76 ($P < 0.0001$), which corresponds to a mean AUC reduction of 37.6% in the combination periods.

Pharmacodynamics. The suppression of serum estrogen levels and effects on SHBG, LH, and FSH compared to baseline are summarized in Table 3. Twelve patients had at least one endocrine measurement available and are included in the analysis. E_1 , E_2 , and E_{1S} levels dropped significantly after baseline, with many values below the detection limits of the respective assays. The mean estrogen levels underestimated the suppressive effects of letrozole because undetectable values were given the value of the assay detection limit for statistical purposes. No

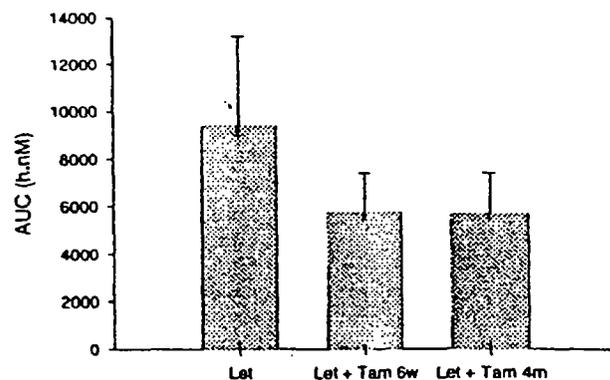


Fig. 2 Comparison of the mean \pm SD AUC for plasma letrozole after 6 weeks of treatment with letrozole alone, 6 weeks of treatment with letrozole plus tamoxifen, and >4 months of treatment with letrozole plus tamoxifen. The ratio of AUC (letrozole alone):AUC (combination, 6 weeks) was 1.60 ($P < 0.0001$).

significant changes were seen after tamoxifen was added at 6 weeks. SHBG, LH, and FSH did not change significantly from baseline during either phase. A slight decrease in LH and FSH levels and an increase in SHBG levels were seen when tamoxifen was added at 6 weeks; however, the sample size was too low to detect a significant difference.

DISCUSSION

This study was conducted largely to ensure that there were no unexpected pharmacokinetic or pharmacological interactions between letrozole and tamoxifen that would preclude their combination in future clinical studies. It has previously been noted that the aromatase inhibitor AG enhanced the clearance of tamoxifen in advanced breast cancer patients (9). AG shows similar interaction with a number of other drugs and is dependent on the induction of CYPs. In contrast, a recent study has reported that letrozole has no impact on the plasma levels of tamoxifen or its major metabolites (12).

The impact of tamoxifen in reducing the plasma AUC of letrozole by 38% on average was unexpected. Despite the very widespread use of tamoxifen over a 20-year period and its application in patient groups that are frequently taking many drugs, no such interaction has been reported previously. It is not known whether tamoxifen interacts with other triazole aromatase inhibitors, e.g., anastrozole, in this way. The extension of this study beyond the core 12 weeks allowed a second profile to be made on combination therapy and showed that this change in pharmacokinetics was not transient, nor did it increase in magnitude during continued therapy. The reduction varied between patients, ranging from no reduction in one patient to a reduction of almost 70%.

The decreased drug levels are estimated to correspond to a daily dose of approximately 1.5–2 mg of letrozole if letrozole were administered alone. The clinical consequences of this interaction have not been studied. The patients still received tamoxifen as standard therapy, and, as discussed above, data from another trial indicate that tamoxifen levels were not affected by letrozole (12). The response rate and durability of

Table 3 Geometric mean serum levels of E₁, E₂, and E₁S and median serum levels of LH, FSH, and SHBG during the core phase of the trial

Analyte	Baseline	Letrozole			Letrozole + Tamoxifen		
		2 weeks	4 weeks	6 weeks	8 weeks	10 weeks	12 weeks
E ₁ (pmol/liter)	34.8	5.3	6.5	6.9	6.6	5.0	3.9
E ₂ (pmol/liter)	65.1	10.1	10.9	11.6	11.8	10.7	10.0
E ₁ S (pmol/liter)	1172.7	46.3	49.8	57.1	73.5	47.8	29.6
LH (IU/liter)	31.0	36.0	34.0	35.0	24.0	23.5	24.5
FSH (IU/liter)	61.0	80.0	92.0	87.0	49.0	54.5	57.0
SHBG (nmol/liter)	49.0	50.5	51.5	48.5	61.5	83.0	94.5

responses in this trial are similar to those observed previously for patients treated with letrozole alone (3, 4), but the small numbers of patients in this study preclude a reliable estimate of antitumor efficacy. In two large Phase IIb/III trials, the dose of 2.5 mg/day letrozole appeared to achieve a longer time to tumor progression than the 0.5 mg/day dose and was superior in terms of survival (3, 4). Thus, the reduced letrozole levels with the combination may not provide the full additive benefit. Unless additional studies demonstrate a clear benefit for the combination aromatase inhibitor/tamoxifen over sequential use of the drugs, sequential application would seem to be preferable for letrozole for letrozole and tamoxifen.

The reason for the reduction in letrozole levels may lie in an alteration of the absorption of letrozole or in a change of its clearance during tamoxifen treatment. The first hypothesis appears to be unlikely because there are no indications in the literature that tamoxifen alters the absorption behavior of other drugs. In addition, letrozole is reasonably soluble, particularly at low pH values, and is rapidly and completely absorbed after oral dosing (17).

Changes in the clearance of letrozole by tamoxifen may be due to a protein binding interaction or an interaction at the level of the enzymes involved in the metabolism of letrozole. Both compounds bind mainly to albumin. However, *in vitro* experiments on selected plasma samples from this study did not show an altered plasma binding of letrozole in the combination periods compared to the treatment with letrozole alone (18). Thus, protein displacement as a cause for the reduction of letrozole levels appears to be very unlikely.

Tamoxifen and letrozole use a common CYP isoenzyme in their major metabolic elimination pathways. Formation of the main metabolite of letrozole is catalyzed by CYP3A4 and CYP2A6 (19). The contribution of each individual isoenzyme to this pathway is not known. The main metabolic transformation of tamoxifen to *N*-desmethyltamoxifen is mediated by CYP3A4 and probably also by CYP2C (20, 21). In animal experiments, tamoxifen has been shown to induce the rat isoenzymes CYP2B1 and CYP3A1 (22, 23) and to increase 6 β - and 16 α -hydroxylation of testosterone in the rat (22). This indicates that enzymes homologous to the human CYP3A family are induced by tamoxifen. A case report in a single patient describes a decrease in the plasma levels of doxepin (a tricyclic antidepressant) during tamoxifen coadministration that may be due to CYP enzyme induction (24). However, apart from this report, tamoxifen has not been described as a CYP inducer in the clinical literature, but it is important to note that systematic studies on drug-drug interactions have apparently not been performed (24).

Despite this rather sparse support from the literature, the hypothesis of an induction of letrozole-metabolizing enzymes (possibly CYP3A4) by tamoxifen remains the most likely one.

Hormone levels (E₁, E₂, and E₁S) dropped significantly as compared to baseline during letrozole monotherapy. The addition of tamoxifen did not cause any significant change to the diminished hormone levels. This suggests that although letrozole levels were reduced by the addition of tamoxifen, the effect of letrozole on hormone levels remains largely unaltered. Any minor alterations are unlikely to be detected because many estrogen levels were below the limit of detection of the assay. Levels of SHBG, LH, and FSH did not change significantly from baseline on the addition of letrozole, as has been reported previously (2). The apparent decreases in LH and FSH levels and increases in SHBG levels on the introduction of tamoxifen are also consistent with previous reports on tamoxifen alone (25). Thus, the pharmacodynamic changes with the two drugs appear to be independent of one another and would not be expected to compromise their activity.

Overall, the addition of tamoxifen did not increase the incidence of adverse events, with the possible exception of weight gain. All side effects reported had previously been reported with letrozole or tamoxifen individually.

These data on reduced letrozole levels may have significant implications beyond the immediate combined usage of these two drugs. The widespread usage of tamoxifen inevitably results in its combined application with a large number of other drugs. The lack of clinical reports of toxic interactions indicates the probable absence of any safety concerns, but it is possible that tamoxifen may unexpectedly interact with other drugs and that these interactions might affect the efficacy of the drugs, particularly in cases where there is a steep dose-response curve.

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Tamoxifen and toremifene concentrations in plasma are greatly decreased by rifampin

Background: Rifampin (INN, rifampicin) is a potent inducer of cytochrome P450 (CYP) enzymes involved in drug metabolism and therefore causes many drug interactions.

Methods: The effects of rifampin on the pharmacokinetics of tamoxifen (study I) and toremifene (study II) were examined in 2 randomized, placebo-controlled crossover studies. Ten (study I) or 9 (study II) healthy male volunteers took either 600 mg rifampin or placebo orally once a day for 5 days. On the sixth day, 80 mg tamoxifen or 120 mg toremifene was administered orally. Blood samples were collected up to 336 hours after drug administration.

Results: Rifampin reduced the area under the plasma concentration-time curve (AUC) of tamoxifen by 86% ($P < .001$), peak plasma concentration (C_{max}) by 55% ($P < .001$), and elimination half-life ($t_{1/2}$) by 44% ($P < .001$). The AUC of toremifene was reduced by 87% ($P < .001$), C_{max} by 55% ($P < .001$), and $t_{1/2}$ by 44% ($P < .01$) with rifampin. During the rifampin phase, the AUC of *N*-demethyltamoxifen was 38% ($P < .001$) and the AUC of *N*-demethyltoremifene was 20% ($P < .01$) of that during the placebo phase.

Conclusions: Rifampin markedly reduces the plasma concentrations of tamoxifen and toremifene by inducing their CYP3A4-mediated metabolism. Concomitant use of rifampin or other potent inducers of CYP3A4 with tamoxifen and toremifene may reduce the efficacy of these antiestrogens. (Clin Pharmacol Ther 1998;64:648-54.)

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Tamoxifen and its chlorinated derivative toremifene are widely used nonsteroidal antiestrogens. The pharmacokinetics of tamoxifen and toremifene are characterized by good absorption, metabolism in the liver, and a very long elimination half-life.¹⁻⁴ The main metabolic pathway of both tamoxifen and toremifene is *N*-demethylation, which is mediated by cytochrome P450 3A4 (CYP3A4).⁵⁻⁷ *N*-Demethyltamoxifen and *N*-demethyltoremifene are biologically active and may contribute to the activity of the parent drug in vivo.

The cytochrome P450 enzymes, which are found both in the liver and in extrahepatic tissues, play a leading role in oxidative drug metabolism. CYP3A4, the

most abundant cytochrome P450 enzyme in human liver, is involved in the biotransformation of a large number of drugs, including several anticancer agents.^{8,9} CYP3A4 can be induced or inhibited by many medications, which makes CYP3A4 substrates susceptible to interactions with other drugs.

Rifampin (INN, rifampicin), which is used, for example, in the treatment of patients with tuberculosis, is a very potent inducer of CYP3A4 and some other cytochrome P450 enzymes.¹⁰ Rifampin markedly reduces the plasma concentrations of many CYP3A4 substrates, such as midazolam, triazolam, verapamil, and cyclosporine (INN, ciclosporin).¹¹⁻¹⁴ However, there seem to be no data on the effects of rifampin on the pharmacokinetics of tamoxifen and toremifene. As CYP3A4 substrates, tamoxifen and toremifene may be susceptible to induction of CYP3A4. The aim of this study was to characterize the effects of rifampin on the pharmacokinetics of these drugs.

METHODS

Subjects. Ten healthy male volunteers (age range, 19 to 32 years; weight range, 52 to 88 kg) participated in the tamoxifen study (study I). Nine healthy male vol-

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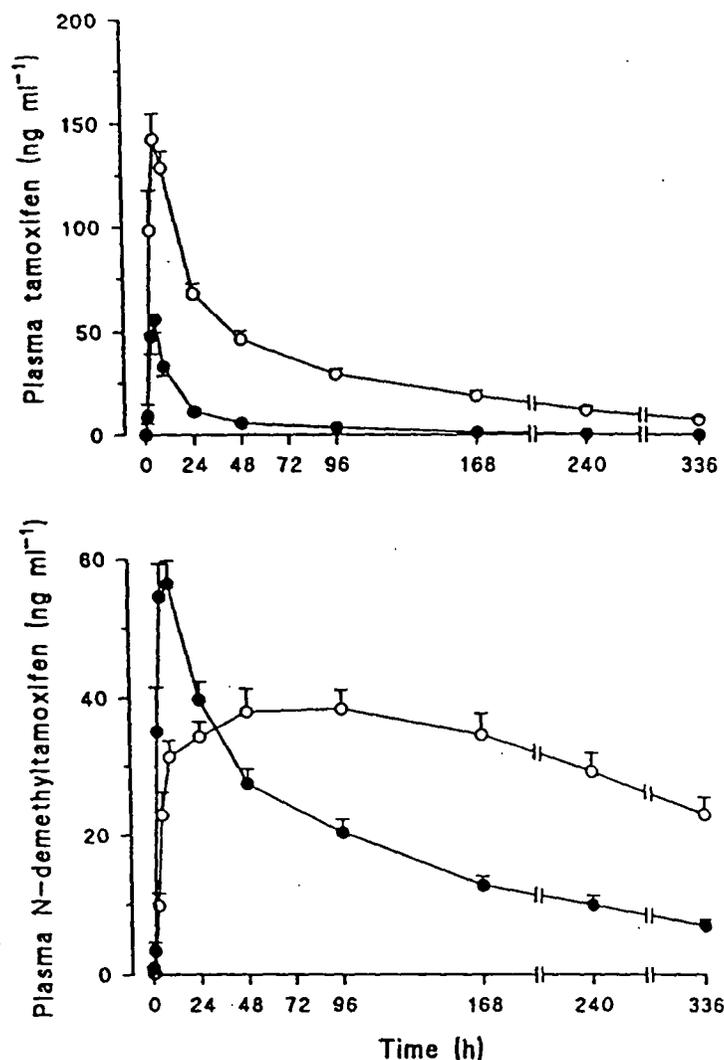


Figure 1. Plasma concentrations of tamoxifen and *N*-demethyltamoxifen in 10 healthy subjects (mean value \pm SEM) after an oral dose of 80 mg tamoxifen after pretreatment with 600 mg rifampin (solid circles) or placebo (open circles) once a day for 5 days.

unteers (age range, 22 to 36 years; weight range, 60 to 87 kg) participated in the toremifene study (study II). The subjects were ascertained to be healthy by means of medical history, physical examination, and routine laboratory tests before they entered the study. None of the subjects took continuous medications. All volunteers gave written informed consent.

Study design. Two separate randomized, placebo-controlled, 2-phase crossover studies were conducted. The phases were separated by a washout period of 6 weeks. The general design was similar for both studies. The subjects took either 600 mg rifampin (Rimapen, Orion Pharma, Espoo, Finland) or matched placebo by

mouth once a day at 8 PM for 5 days. On the sixth day, 80 mg tamoxifen in two 40-mg tablets (Nolvadex, Zeneca Pharmaceuticals, Cheshire, England) (study I) or 120 mg toremifene in two 60-mg tablets (Fareston, Orion Pharma, Turku, Finland) (study II) was administered orally with 150 mL water at 1 PM, that is, 17 hours after the last dose of rifampin. The relatively high doses of tamoxifen and toremifene were chosen to allow the determination of plasma drug concentrations even if they were greatly lowered by rifampin.

The subjects fasted for 2 hours before administration of the antiestrogen. A light standard meal was served at 4 PM and 7 PM. Alcohol and smoking were not

Table I. The pharmacokinetic variables of 80 mg tamoxifen and *N*-demethyltamoxifen among 10 subjects after pretreatment with placebo or 600 mg rifampin once a day for 5 days

Variable	Placebo phase	Percentage of control value	Rifampin phase	Percentage of control value	P Value
<i>Tamoxifen</i>					
C_{max} (ng · mL ⁻¹)	145 ± 12	100	64 ± 5.0	45 (24-62)	<.001
t_{max} (h)	5 (5-9)		4 (3-9)		<.05
$t_{1/2}$ (h)	118 ± 10	100	68 ± 13	56 (21-91)	<.001
AUC (μg · mL ⁻¹ · h)	10.7 ± 1.1	100	1.46 ± 0.18	14 (9-18)	<.001
<i>N-Demethyltamoxifen</i>					
C_{max} (ng · mL ⁻¹)	41 ± 3.0	100	60 ± 3.6	149 (111-181)	<.001
t_{max} (h)	96 (9-240)		5 (3-9)		<.01
$t_{1/2}$ (h)	329 ± 53	100	198 ± 37	64 (41-113)	<.01
AUC (μg · mL ⁻¹ · h)	22.9 ± 3.7	100	8.14 ± 1.0	38 (22-51)	<.001

Data are mean values ± SEM; median values and ranges are given for t_{max} . Values in parentheses are ranges. C_{max} , Peak plasma concentration; t_{max} , time to reach C_{max} ; $t_{1/2}$, half-life; AUC, area under the plasma concentration-time curve.

allowed on the study days. The study protocol was approved by the ethics committee of the Department of Clinical Pharmacology, University of Helsinki, and the Finnish National Agency for Medicines.

Blood sampling and determination of plasma drug concentrations. Timed blood samples were drawn from a forearm vein just before the antiestrogen was administered and 1, 3, 5, 9, 24, 48, 96, 168, 240, and 336 hours later. The blood samples (10 mL each) were drawn into tubes that contained ethylenediaminetetraacetic acid. Plasma was separated within 30 minutes and stored at -20°C or colder until analysis. Plasma tamoxifen, *N*-demethyltamoxifen, toremifene, and *N*-demethyltoremifene concentrations were determined by means of HPLC.¹⁵ The limit of quantitation was 2 ng · mL⁻¹ for tamoxifen and toremifene, and 1 ng · mL⁻¹ for *N*-demethyltamoxifen and *N*-demethyltoremifene. The interassay coefficient of variation was less than 10% for all compounds at relevant concentrations.

Pharmacokinetics. The pharmacokinetics of tamoxifen, *N*-demethyltamoxifen, toremifene, and *N*-demethyltoremifene were characterized by peak concentration in plasma (C_{max}), time to C_{max} (t_{max}), total area under the plasma concentration-time curve (AUC), and elimination half-life ($t_{1/2}$). The terminal log-linear phase of the plasma concentration-time curve was identified visually for each subject. The elimination rate constant (k_e) was determined by means of linear regression analysis with use of the last 3 to 6 points on the plot of the natural logarithm of the plasma concentration-time curve. The $t_{1/2}$ value was calculated from the equation $t_{1/2} = \ln 2 \cdot k_e^{-1}$. The AUC values were calculated with the trapezoidal rule with extrapolation to infinity by means of dividing the last measured concentration by the k_e value.

Statistical analysis. Results are expressed as mean values ± SEM or, in case of the t_{max} , as median value with range. The pharmacokinetic variables after the 2 pretreatments, rifampin and placebo, were compared with a paired *t* test (2-tailed). The Wilcoxon test was used for analysis of t_{max} values. The level of statistical significance was $P < .05$.

RESULTS

Rifampin substantially reduced the plasma concentrations of tamoxifen and toremifene and their active metabolites *N*-demethyltamoxifen and *N*-demethyltoremifene.

Pharmacokinetics of tamoxifen and *N*-demethyltamoxifen (study I). Rifampin reduced the AUC of tamoxifen by 86% (from 10.7 ± 1.1 μg · mL⁻¹ · h to 1.46 ± 0.18 μg · mL⁻¹ · h; $P < .001$), C_{max} value by 55% (from 145 ± 12 ng · mL⁻¹ to 64 ± 5.0 ng · mL⁻¹; $P < .001$), and $t_{1/2}$ value by 44% (from 118 ± 10 hours to 68 ± 13 hours; $P < .001$) (Figure 1 and Table I). The AUC of *N*-demethyltamoxifen during the rifampin phase was 38% of that during the placebo phase ($P < .001$). The t_{max} of *N*-demethyltamoxifen occurred earlier during the rifampin phase (5 hours [median]) than during the placebo phase (96 hours), and the C_{max} value was about 50% higher after rifampin treatment (60 ± 3.6 ng · mL⁻¹ versus 41 ± 3.0 ng · mL⁻¹; $P < .001$). The $t_{1/2}$ value of *N*-demethyltamoxifen decreased 36% (from 329 ± 53 hours to 198 ± 37 hours; $P < .01$) with rifampin (Figure 1 and Table I).

Pharmacokinetics of toremifene and *N*-demethyltoremifene (study II). After rifampin (Figure 2; Table II) the AUC of toremifene decreased 87% (from 34.1 ± 4.7 μg · mL⁻¹ · h to 4.43 ± 0.75 μg · mL⁻¹ · h; $P < .001$).

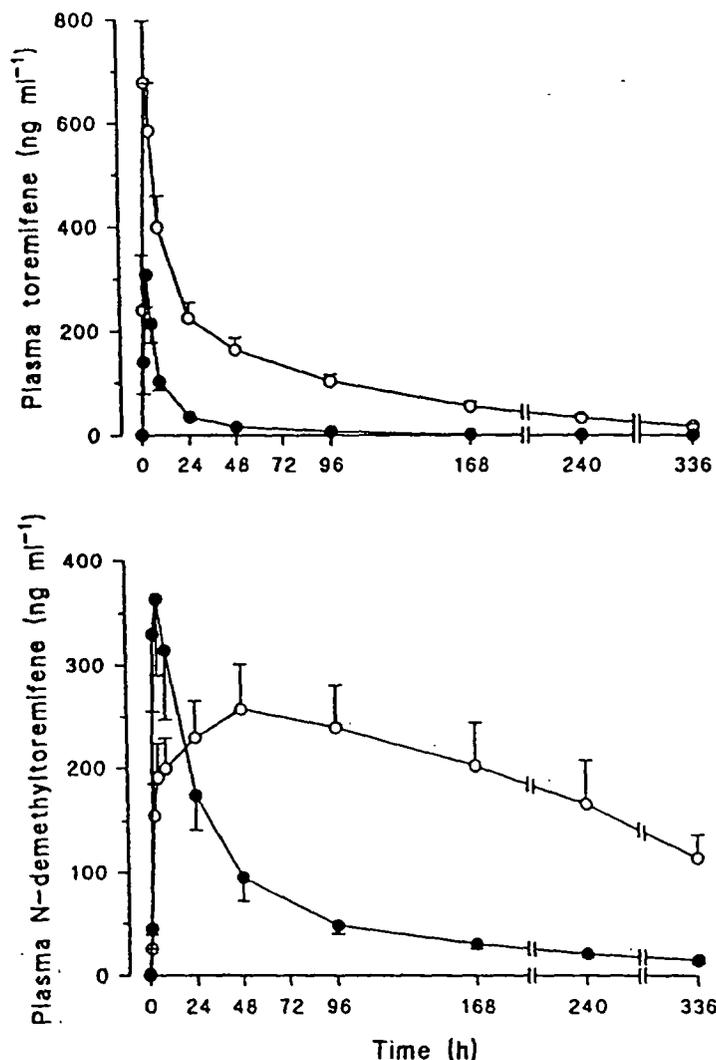


Figure 2. Plasma concentrations of toremifene and *N*-demethyltoremifene in 9 healthy subjects (mean value \pm SEM) after an oral dose of 120 mg toremifene after pretreatment with 600 mg rifampin (solid circles) or placebo (open circles) once a day for 5 days.

The C_{max} value was reduced 55% (from 722 ± 112 ng \cdot mL⁻¹ to 322 ± 59 ng \cdot mL⁻¹; $P < .001$). The $t_{1/2}$ value was reduced 44% (from 99 ± 8.3 hours to 53 ± 10 hours; $P < .01$). During the rifampin phase, the AUC of *N*-demethyltoremifene was 20% of that during the placebo phase ($P < .01$). Rifampin increased the C_{max} of *N*-demethyltoremifene 48% (391 ± 74 ng \cdot mL⁻¹ versus 267 ± 43 ng \cdot mL⁻¹; $P < .01$) and shortened the t_{max} value (5 hours versus 48 hours). The $t_{1/2}$ value of *N*-demethyltoremifene decreased 41% (from 287 ± 54 hours to 148 ± 17 hours; $P < .05$) (Figure 2 and Table II).

DISCUSSION

The results of this study showed that even short-term use of rifampin greatly reduces the plasma concentrations of tamoxifen and toremifene. The effects of rifampin on the pharmacokinetics of tamoxifen and toremifene were very similar. After rifampin treatment, the AUC of tamoxifen was only 14% and the AUC of toremifene only 13% of that after placebo treatment. The C_{max} values of tamoxifen and toremifene decreased about 50% with rifampin treatment, suggesting that the presystemic metabolism of these drugs increased. The

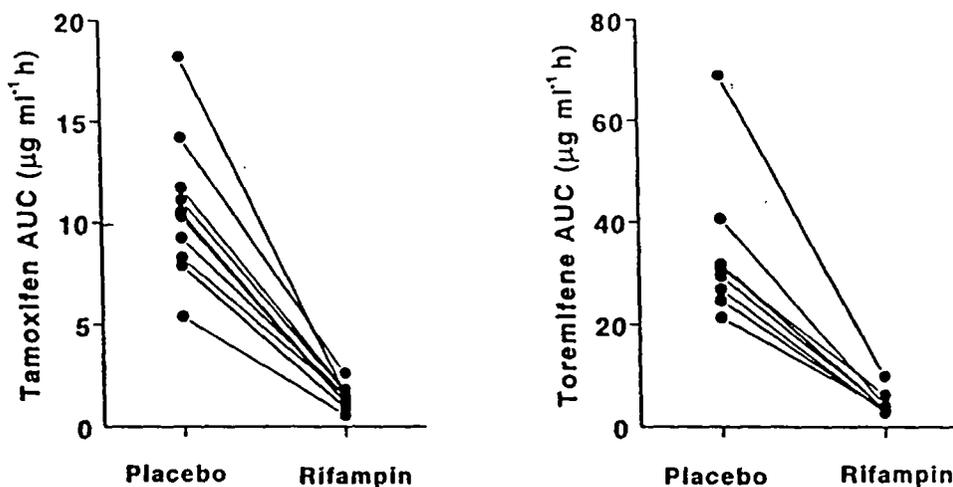


Figure 3. Individual values of the total area under the plasma concentration–time curve (AUC) of 80 mg tamoxifen and 120 mg toremifene during the placebo and rifampin phases.

Table II. Pharmacokinetic variables of 120 mg toremifene and *N*-demethyltoremifene in 9 subjects after pretreatment with placebo or 600 mg rifampin once a day for 5 days

Variable	Placebo phase	Percentage of control value	Rifampin phase	Percentage of control value	P Value
<i>Toremifene</i>					
C_{max} (ng · mL ⁻¹)	722 ± 112	100	322 ± 59	45 (27-52)	<.001
t_{max} (h)	3 (1-5)		3 (1-5)		.32
$t_{1/2}$ (h)	99 ± 8.3	100	53 ± 10	56 (13-98)	<.01
AUC (µg · mL ⁻¹ · h)	34.1 ± 4.7	100	4.43 ± 0.75	13 (10-20)	<.001
<i>N</i> -Demethyltoremifene					
C_{max} (ng · mL ⁻¹)	267 ± 43	100	391 ± 74	148 (107-189)	<.01
t_{max} (h)	48 (24-96)		5 (3-5)		<.01
$t_{1/2}$ (h)	287 ± 54	100	148 ± 17	59 (30-83)	<.05
AUC (µg · mL ⁻¹ · h)	114.7 ± 21.4	100	22.9 ± 4.5	20 (16-27)	<.01

Data are mean values ± SEM; median values and ranges are given for t_{max} . Values in parentheses are ranges. C_{max} , Peak plasma concentration; t_{max} , time to reach C_{max} ; $t_{1/2}$, half-life; AUC, area under the plasma concentration–time curve.

decrease in $t_{1/2}$ value of tamoxifen and toremifene caused by rifampin indicated that systemic clearance of tamoxifen and toremifene increased (one assumes that the volume of distribution remained unaltered). There was only moderate variability between subjects in the magnitude of these interactions, and the interaction was striking for all subjects (Figure 3).

The AUC of the principal active metabolites of tamoxifen and toremifene, *N*-demethyltamoxifen and *N*-demethyltoremifene, was lowered considerably with rifampin treatment. However, the C_{max} of *N*-demethyltamoxifen and *N*-demethyltoremifene was higher and occurred earlier after rifampin treatment than after placebo treatment. These changes probably were

caused by the enhanced presystemic metabolism of the parent drug and reflect an increased rate of formation of the metabolites. That the $t_{1/2}$ value of *N*-demethyltamoxifen and *N*-demethyltoremifene was decreased significantly suggests that the oxidative metabolism of these compounds was increased with rifampin.

The CYP3A4-inducing effect of rifampin probably was not yet maximal at the time of administration of tamoxifen or toremifene. Results of one study¹⁶ suggested that about half of the maximal inducing effect was attained during the 5-day pretreatment with rifampin used in our study. Thus the magnitude of the observed interactions might have been even greater after longer use of rifampin. We used a relatively short period

of rifampin treatment because we wanted to expose the volunteers to a minimum amount of rifampin.

Tamoxifen and toremifene are extensively metabolized in the liver, and CYP3A4 is the principal cytochrome P450 enzyme involved in their biotransformation.⁵⁻⁷ Large quantities of CYP3A4 are found in the liver and in small-intestinal enterocytes¹⁷; both the liver and intestine play an important role in the presystemic metabolism of many CYP3A4 substrates. Rifampin is a potent inducer of CYP3A4 in both these organs.^{10,17} The pharmacokinetics of many CYP3A4 substrates undergoing marked presystemic metabolism (often reflected in decreased bioavailability) are highly susceptible to induction (or inhibition) of CYP3A4. The absolute bioavailability of tamoxifen and toremifene is not known, but our results—decreased C_{max} and shorter $t_{1/2}$ values—indicated that rifampin enhanced the metabolism of tamoxifen and toremifene during both presystemic metabolism and the elimination phase.

Our results are in good agreement with those of other studies of the effect of rifampin on the pharmacokinetics of CYP3A4 substrates that undergo extensive presystemic metabolism.¹¹⁻¹⁴ For example, rifampin greatly reduces the plasma concentrations and effects of oral midazolam and triazolam, 2 benzodiazepine hypnotics metabolized predominantly by CYP3A4. In these studies, the AUC of midazolam and triazolam after rifampin treatment was only about 5% of that after placebo.^{12,14} With midazolam, a very similar interaction occurred in patients receiving a CYP3A4-inducing antiepileptic agent (carbamazepine or phenytoin).¹⁸

Some studies have shown that drugs that cause enzyme induction can lower the plasma concentrations of these antiestrogens. Aminoglutethimide markedly reduced the concentrations of tamoxifen and most of its metabolites.¹⁹ In another study, patients with glioma who received phenytoin with high-dose tamoxifen showed a trend toward lower concentrations of tamoxifen than patients without phenytoin.²⁰ The AUC of toremifene was 50% smaller among patients receiving carbamazepine, phenobarbital, or phenytoin than among healthy control subjects.¹⁵

In conclusion, rifampin substantially reduces the plasma concentrations of tamoxifen and toremifene by inducing their CYP3A4-mediated metabolism. Although the clinical significance of these interactions is not clear from this study, it is reasonable to assume that rifampin and other potent inducers of CYP3A4, such as carbamazepine and phenytoin, may reduce the therapeutic efficacy of these antiestrogens. The dosage of tamoxifen and toremifene should be considerably increased during concomitant use of rifampin or other CYP3A4-inducing agents.

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