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*Perspective*

# Is There a Case for P-450 Inhibitors in Cancer Treatment?

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In mammals, the cytochrome P-450 monooxygenase enzyme system (P-450) is involved in the synthesis and/or degradation of a large number of endogenous compounds and in the biotransformation of drugs and other xenobiotics. The list of "P-450-sensitive" endogenous compounds has become quite impressive, comprising molecules as diverse as cholesterol, steriod hormones, vitamins, and eicosanoids. The key role that some of these endobiotics play—or are thought to play—in (patho)physiological processes makes the P-450 system a logical target for drug development.

This paper will deal with the applicability of P-450 inhibition as a chemotherapeutic strategy in the struggle against cancer.

## Cytochrome P-450 Enzyme System

The P-450 enzyme system consists of a large and ubiquitous family of medium-size proteins (molecular weight between 50 and 60 kD) that contain a single iron protoporphyrin IX prosthetic group.[1] Thanks to gene cloning and DNA sequencing, the number of distinct eukaryotic P-450 enzymes known today exceeds 60.[2] In mammals, P-450 enzymes occur most abundantly in the liver, but they are present in virtually all tissues except in serum, striated muscle, neurons, and red blood cells.[3] Their subcellular localization is the lipophilic membrane of the endoplasmatic reticulum (microsomes) or of the inner mitochondrial membrane.[1]

Mammalian P-450 enzymes share some common enzymatic and molecular properties. Enzymatically, most of them are versatile monooxygenases, able to insert one oxygen atom of the $O_2$ molecule into a large number of substrates, while reducing the other oxygen atom by two electrons to $H_2O$, according to the following equation:[1,4]

$$R\text{–}H + O_2 + 2H^+ + 2e^- \xrightarrow{\text{P-450}} R\text{–}OH + H_2O$$

Besides hydroxylating alkanes to alcohols, some P-450 enzymes also convert alkenes to epoxides, arenes to phenols, and sulfides to sulfoxides and sulfones or oxidatively split C–N, C–O, C–C, or C–S bonds.

P-450 enzymes are believed to monooxygenate via a common pathway,[4] departing from the enzyme in its ground state, i.e. with the heme in the six-coordinated low-spin $Fe^{III}$ conformation (Figure 1, **1**). A water molecule (or the hydroxyl group from a seryl, tyrosyl, or threonyl residue) occupies the axial sixth coordination position opposite to the cysteinate fifth ligand.[5] Binding of the substrate (RH) perturbs the enzyme resulting in the loss of the sixth heme ligand and in the formation of the five-coordinated high-spin $Fe^{III}$ state (**2**), which has a higher reduction potential than the low-spin configuration. After accepting one electron, the substrate-bound enzyme is reduced to the five-coordinated high-spin $Fe^{II}$ configuration (**3**), thereby gaining the ability to coordinate one oxygen molecule as the sixth ligand to its heme iron atom to form a ferric–oxy intermediate (**5**). Transfer of a second electron reduces this species to a ferric–peroxy state (**6**). Cleavage of the O–O bond, facilitated by the strong electron pushing capabilities of the cysteinate fifth ligand results in the release of water and the formation of a ferryl–oxy intermediate (**7**). The oxygen atom in this state, containing only seven valence electrons, exerts a strong electrophilic activity and abstracts an hydrogen atom or electron from the nearby bound substrate. This abstraction yields a substrate radical and an $Fe^{III}$-bound hydroxyl radical. Both radical species then readily combine to an hydroxylated product.

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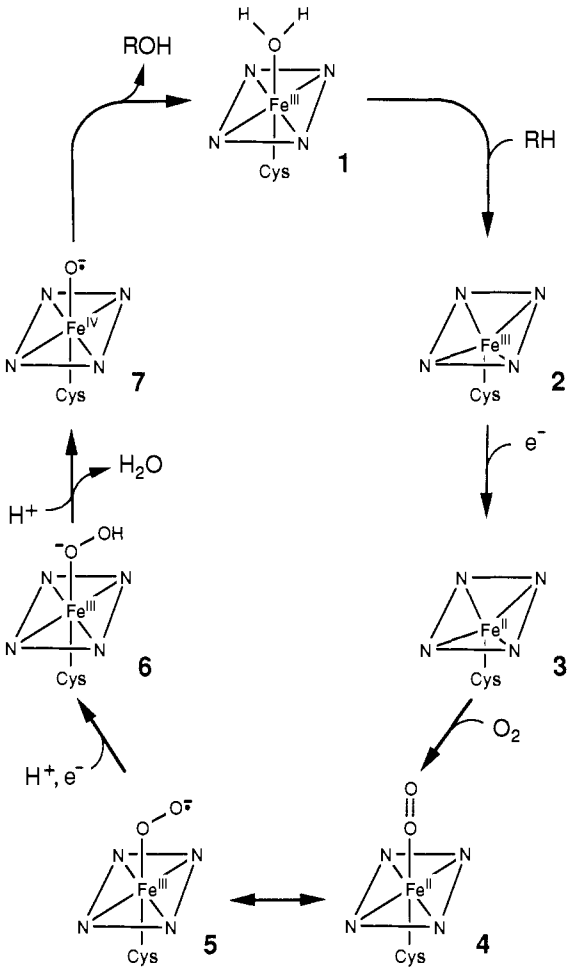


**Figure 1.** The catalytic cycle of cytochrome P-450. The dianionic porphyrin complex is represented by a parallelogram with nitrogen atoms at each corner.[4] Intermediates 6 and 7 are hypothetical structures.

During all P-450 reactions, the obligatory electrons are generated by auxiliary enzyme systems that involve NADPH and an NADPH-cytochrome P-450 reductase (in endoplasmic reticulum), or NADH, ferredoxin, and a flavoprotein reductase (in mitochondria).[3] Carbon monoxide (CO) competes with oxygen to occupy the free, sixth ligand site of the pentacoordinated high-spin $Fe^{II}$ configuration (Figure 1, 3). Not surprisingly, CO inhibits P-450 enzyme activity, and, indeed, sensitivity toward CO inhibition is considered one of the main characteristics of P-450 enzymes. Upon binding to CO, P-450 enzymes adopt a six-coordinated low-spin $Fe^{II}$ state and display a difference spectrum characterized by absorption maxima at 450 nm, instead of the 420 nm observed with other hemoprotein–CO complexes. The occurrence of the absorption maximum at 450 nm led to the designation P (for pigment)-450 for these enzymes.[6]

Molecularly, P-450 enzymes are all coded by genes that are derived from one ancestral gene.[2,7] As such, all known P-450 genes can be grouped into about 10 different families and further subdivided into 15 or so subfamilies. Amino acid sequences within a family are ≥36% similar, while sequences within subfamilies match for more than 70%. Although the overall sequence analogy between P-450 families is not very significant, it is possible to discern some domains of sequence similarity, each possessing its own pattern of invariant, conserved, and variable amino acids. One such domain, localized near the carboxyl end of the enzyme, is about 25 amino acids long and contains an invariant cysteine that provides the fifth axial ligand to the heme iron atom. Also reminiscent of a common ancestry is the observation that the expression of activity of many P-450 enzymes is susceptible to a number of similar extracellular stimuli. Certain chemicals (barbiturates, polycyclic aromatic compounds, ...) enhance or suppress tissue levels of several individual P-450 enzymes through processes that involve altered production, transcription, or stability of P-450 messenger RNAs.[8]

## P-450 Enzyme Inhibition

In theory, every step along the P-450 catalytic pathway is a potential target for drug inhibition. In practice, only three steps have been found vulnerable to interaction by drugs:[5,9] (i) the binding of the substrate, (ii) the binding of molecular oxygen, and (iii) the transfer of oxygen to the substrate. However, interference with only one of these steps does not lead to sufficient or acceptable inhibition. Blocking of P-450 activity by competition with substrate binding demands an inhibitory substance that is avidly bound but has poor substrate characteristics: two properties that have been found difficult to reconcile within one molecule.[9] Otherwise, inhibition of oxygen binding by competitive coordination to ferrous/ferric heme should be regarded as theoretical: only CO, cyanide, and isocyanides function in this way.[9] More or less all effective P-450 inhibitors known today possess the capacity to interfere with two steps of the enzymatic process. As such, three not mutually exclusive types of P-450 inhibitors can be defined: (i) Compounds that, in a competitive and reversible manner, interact with the substrate binding site *and* coordinate with the heme iron atom. (ii) Compounds that bind as "normal" substrates but are catalytically converted by the enzyme into a reactive intermediate which then alkylates a proximal nucleophilic residue of the enzyme, causing its inactivation. Such irreversible inhibitors are also known as mechanism-based enzyme inhibitors or suicide substrates.[11] Compounds of categories i and ii are termed Type I inhibitors: upon interaction with a P-450 enzyme, they usually produce a so-called Type I difference spectrum characterized by an absorption maximum at about 420 nm and an absorption minimum at 392 nm.[10] (iii) Compounds that interact, as the sixth ligand, with the heme atom *and* combine with amino acid residues located close to the heme site. This reversible interaction is optimally performed by compounds that carry a heterocyclic nitrogen atom possessing a free electron pair for coordination with the heme atom (e.g. imidazole, triazole, pyridine, pyrimidines), *and* a substituent for interaction with nearly located protein residues. Binding of such inhibitors to a P-450 enzyme gives rise to a Type II difference spectrum with an absorption maximum at about 430 nm.[12] At least in vitro, three aspects determine the strength and selectivity of these Type II inhibitors: the nature of the nitrogen-containing heterocyclic moiety, the probability of hydrogen bond and/or van der Waals interactions by the heterocyclic substituent with the enzyme,

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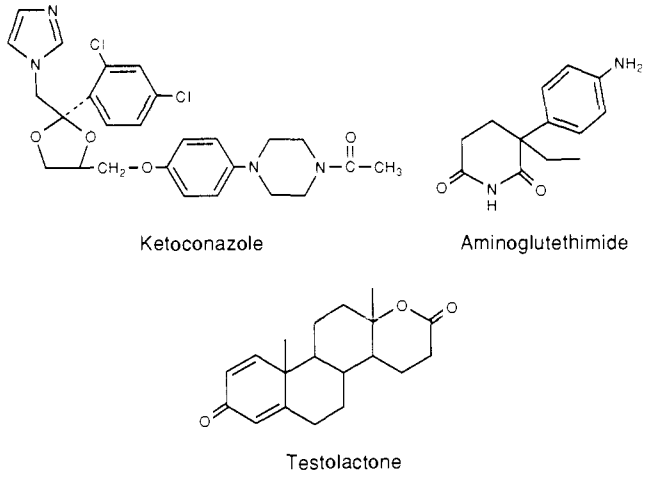


**Figure 2.** Chemical structures of aminoglutethimide or 3-(4-aminophenyl)-3-ethyl-2,6-piperidinedione, ketoconazole or 1-acetyl-4-[4-[[2-(2,4-dichlorophenyl)-2-(1*H*-imidazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy]phenyl]piperazine, and testolactone or D-homo-17$\alpha$-oxaandrosta-1,4-diene-3,17-dione.

and the degree of compatibility or synergism between binding to the heme iron and interaction with the protein residues.

A respectable number of Type II P-450 inhibitors have developed into well-known therapeutic agents. More than 15 1-substituted imidazoles and 1,2,4-triazoles, such as miconazole, clotrimazole, tioconazole, ketoconazole, itraconazole, fluconazole, have become highly effective topical and/or systemic antifungal drugs in human medicine.[13] It is now common knowledge that these compounds derive their activity from the inhibition of the biosynthesis of ergosterol, the major sterol in fungal membranes. More in particular, they block the P-450-dependent 14$\alpha$-demethylation of 24-methylenedihydrolanosterol to ergosterol, which results in accumulation of 14$\alpha$-methylsterols and disruption of the fungal cell membrane.[13-15]

On the contrary, only three P-450 inhibitors have been used in human cancer therapy: the Type II inhibitors ketoconazole and aminoglutethimide (AG) and the Type I inhibitor testolactone. The chemical structures of these compounds are depicted in Figure 2. A common denominator between these drugs is their ability to block crucial P-450-dependent steps in the production and metabolism of steroid hormones in mammals. Their therapeutic use is therefore restricted to the treatment of steroid-sensitive or steroid-producing cancers, such as prostate, breast, and adrenal carcinomas.

## Prostate Carcinoma

The term prostate carcinoma refers to neoplasms that originate in the parenchymal epithelium of the prostate. An estimated three-quarters of prostatic cancer is androgen-sensitive, i.e. male sex hormones, in particular 5$\alpha$-dihydrotestosterone, the reduced metabolite of testosterone, mediate growth and development of the cancerous tissue.[16] Suppression of release, synthesis, or actions of

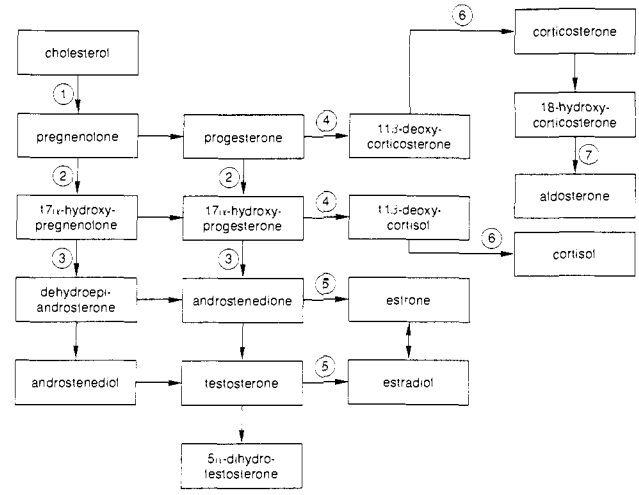


**Figure 3.** Main pathways of steroidogenesis in humans. Numbered arrows indicate reaction steps that are carried out by P-450 enzymes: step 1, desmolase or cholesterol side-chain cleavage enzyme; step 2, 17$\alpha$-hydroxylase; step 3, 17,20-lyase; step 4, 21-hydroxylase; step 5, aromatase; step 6, 11$\beta$-hydroxylase; step 7, 18-hydroxylase.

androgenic hormones has become a major therapeutic option in the management of this malignancy. The consideration that the biosynthesis of testicular and adrenal androgens depends on the action of P-450 enzymes[17] makes inhibition of these enzymes a valuable technique to lower or, if possible, to eliminate the availability of androgens to the neoplastic prostate organ.

Figure 3 gives a schematic representation of the main biosynthetic pathways of steroids in humans.[17] The bioconversion of cholesterol to testosterone and 5$\alpha$-dihydrotestosterone proceeds via two routes: one involving 5-ene 3$\beta$-hydroxysteroids such as pregnenolone and dehydroepiandrosterone, the other involving 4-ene 3-oxosteroids such as progesterone and 4-androstenedione. Both routes are responsible for the mass reduction of the $C_{21}$ steroids pregnenolone and progesterone to form a series of androgenic $C_{19}$ steroids. The crucial steps of this process are mediated by one P-450 protein that displays two enzymatic activities:[18] 17$\alpha$-hydroxylase (Figure 3, step 2), which stereospecifically hydroxylates pregnenolone and progesterone at $C_{17}$, and 17,20-lyase (step 3), which catalyzes the side-chain cleavage of the 17-hydroxylated derivatives of pregnenolone and progesterone. Obviously, effective inhibition of 17$\alpha$-hydroxylase and/or 17,20-lyase activity would produce a sharp decrease of plasma and tissue concentrations of androgenic steroids.

Currently, AG and ketoconazole are the only P-450 inhibitors that have been given to a sufficient number of prostate cancer patients to allow meaningful evaluation of the clinical data.

## Aminoglutethimide

AG was introduced in the early sixties as an anticonvulsant. Clinical activity, however, was disappointing and the compound was eventually degraded to a second-line medication.[19] Moreover, side effects such as rash, dizziness, headache, and drowsiness were all too frequently observed. Prolonged treatment for several months re-

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vealed hormonal imbalance in a few patients, who showed signs of adrenal insufficiency. As a result, in 1966, AG was withdrawn but promptly reformulated to explore its endocrine effects. In vitro experiments indicated that AG inhibited several P-450-dependent adrenal steroidogenic enzymes.[19,20] In decreasing order of inhibition sensitivity these enzymes were as follows: 18-hydroxylase (in Figure 3, step 7; conversion of corticosterone to aldosterone) > desmolase or cholesterol side-chain cleavage enzyme (step 1; conversion of cholesterol to pregnenolone) > 11$\beta$-hydroxylase (step 6; hydroxylation of 11-deoxycortisol) > 21-hydroxylase (step 4; hydroxylation of 17$\alpha$-hydroxyprogesterone).

In vivo animal studies and clinical trials in humans supported the in vitro findings. Treatment with AG lowered circulating concentrations of aldosterone and, to a lesser extent, of cortisol, but enhanced tissue levels of cholesterol.[20] Given its ability to inhibit adrenal steroidogenesis, AG was thought capable to elicit a "medical" adrenalectomy (as opposed to surgical adrenalectomy) and, as such, to be clinically valuable in orchiectomized cancer patients whose adrenals remain the only source of androgen production. Clinically, AG (1000 mg/day), supplemented with hydrocortisone (40 mg/day) or cortisone acetate (25 mg/day) (mostly to compensate for the inhibition of adrenal cortisol production), was given to about 250 relapsed, orchiectomized patients.[19,21-25] Objective response rates were between 19 and 44% and a high incidence of side effects, especially lassitude and depression, was noticed.

## Ketoconazole

This drug was launched in the late seventies as an orally active, broad spectrum antifungal. The standard dose of 200 mg/day only infrequently produced side effects.[19] However, increasing the dosage to 400–1200 mg/day for prolonged periods of time, as was considered[26] necessary for treatment of disseminated coccidioimycosis (valley fever) or central nervous system infections, revealed ketoconazole's side effects on human steroidogenesis. Under these high dose regimen, a limited number of patients developed gynecomastia or breast tenderness.[27,28] The same side effect was also noticed in two patients treated with 200 mg daily.[27] These clinical observations triggered an intense research effort to unravel the influence of ketoconazole on the hormonal household. The most instructive findings were the following: in normal volunteers, a 200-mg dose of ketoconazole was found to produce a transient decrease of plasma testosterone and 4-androstenedione levels within 4–8 h, which had returned to control values 24 h after drug treatment.[29] The finding[29,30] that androgen inhibition coincided with an increase of plasma 17$\alpha$-hydroxyprogesterone levels pointed to a suppressive effect of ketoconazole on 17,20-lyase activity, a conclusion that was proven correct by subsequent in vitro experiments using testis microsomes and Leydig cell cultures.[31,32]

Strong and persistent suppression of 17,20-lyase activity and, hence, of circulating testosterone levels was achieved by increasing both the dose and frequency of administration of ketoconazole to 400 mg every 8 h.[33,34] This so-called high dose (HD) ketoconazole regime resulted in a sustained lowering of circulating androgen and was therefore considered to be a valuable therapeutic option for patients with advanced prostatic cancer who had become refractory to orchiectomy or other hormonal manipulations.

The clinical results obtained with HD ketoconazole can be summarized as follows: the drug was administered in an open multicenter study to more than 400 patients with metastasized prostatic cancer.[35,36] Nearly half (i.e. 195) of these patients had not been treated previously (naive patients), whereas 219 had relapsed on a previous hormonal treatment (relapsed patients). In both virgin and noncastrated relapsed patient groups, serum testosterone levels almost dropped to castrate range within the first week of treatment. Elevated prostatic acid phosphatase levels normalized within 2 months. Moreover, the performance status of 75% of the patients improved and bone pain was markedly reduced as judged by reduced intake of narcotic analgesics. The objective response rates after 3 and 6 months of treatment were 83 and 71% (naive patients) and 58 and 34% (relapsed patients). During treatment, an expected increase of progestin levels was seen, whereas plasma cortisol and cholesterol levels remained within the normal range. By far the most frequent side effects of HD ketoconazole treatment was gastrointestinal intolerance, seen in about 1/3 of the patients.

**R 75 251 [5-[(3-Chlorophenyl)(1*H*-imidazol-1-yl)-methyl]-1*H*-benzimidazole].** All in all, neither AG nor HD ketoconazole proved to be ideal drugs for prostate cancer treatment. Recently, the development of HD ketoconazole in the treatment of prostatic cancer was stopped, because of the high frequency of gastrointestinal complaints and uncomfortable intake schedule (3 times/day every 8 h). Nevertheless, the clinical experience gained with HD ketoconazole had indicated the therapeutic potential of specific 17,20-lyase and/or 17$\alpha$-hydroxylase inhibitors in the treatment of prostatic carcinoma.

The development of R 75 251 or 5-[(3-chlorophenyl)-(1*H*-imidazol-1-yl)methyl]-1*H*-benzimidazole (Figure 4, 1) represented a first move toward this goal.[37] In dogs, 1 (2.5 mg/kg) reduced plasma testosterone and androstenedione concentrations to castrate levels for at least 12 hours.[38]

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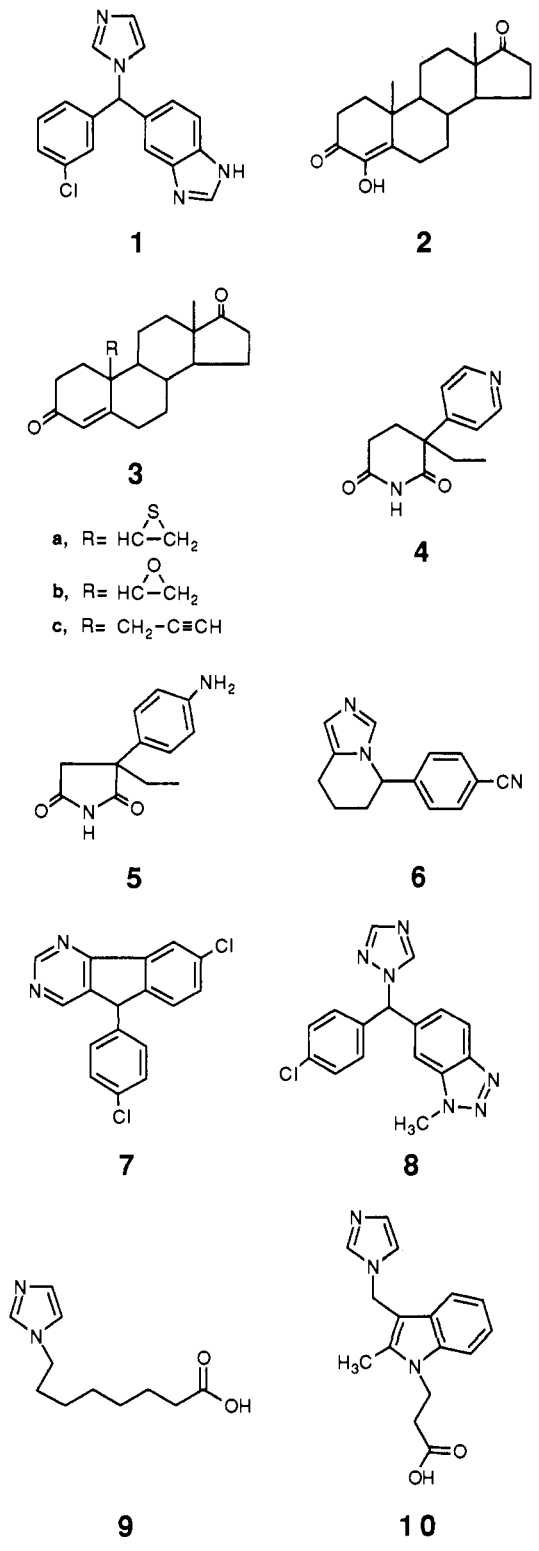


**Figure 4.** Chemical structures of R 75 251 or 5-[(3-chlorophenyl)(1*H*-imidazol-1-yl)methyl]-1*H*-benzimidazole (**1**); 4-hydroxyandrostene-3,17-dione (**2**); 10$\beta$-thiiranylestr-4-ene-3,17-dione (**3a**); 10$\beta$-oxiranylestr-4-ene-3,17-dione (**3b**); MDL 18,962 or 10-(2-propynyl)-estr-4-ene-3,17-dione (**3c**); 3-ethyl-3-(4-pyridyl)piperidine-2,6-dione (**4**); 3-(4-aminophenyl)-3-ethylpyrrolidine-2,5-dione (**5**); CGS 16949A or 4-(5,6,7,8-tetrahydroimidazo[1,5-*a*]pyridin-5-yl)benzonitrile (**6**); LY 113174 or 8-chloro-5-(4-chlorophenyl)-5*H*-indeno[1,2-*d*]pyrimidine (**7**); R 76 713 or 6-[(4-chlorophenyl)(1*H*-1,2,4-triazol-1-yl)methyl]-1-methyl-1*H*-benzotriazole (**8**); OKY 1553 or 1-(7-carboxyheptyl)-imidazole (**9**); dazmegrel or 3-(1*H*-imidazol-1-ylmethyl)-2-methyl-1*H*-indole-1-propanoic acid (**10**).

This reduction was accompanied by accumulation of progesterone, pregnenolone, and 17$\alpha$-hydroxyprogesterone, indicating a combined inhibition of 17$\alpha$-hydroxylase and 17,20-lyase activities. In both male rats and nude mice, 1 (20 mg/kg) inhibited the growth of G-Dunning prostate tumors. In male volunteers, a single oral 300-mg dose of R 75 251 induced gradual decrease of plasma testosterone levels with a nearly complete reduction 8 h after intake.[39]

## Breast Carcinoma

About one-third of breast carcinoma cases are estrogen-dependent, i.e. physiological concentrations of estrogens, in particular estradiol and estrone, maintain and eventually enhance the growth of this malignancy. All endocrine therapies of breast cancer have a common goal: depriving the cancerous tissue of estrogens in the hope of slowing down cancer cell proliferation, increasing cell death and inducing tumor shrinkage.

Estrogens are produced from the androgens androstenedione and testosterone by a P-450-dependent aromatase (Figure 3, step 5).[17] As depicted in Figure 5, aromatase activity involves three separate hydroxylation steps, two at C-19 and one at the C-2 position of the androgen molecule.[17,40] The resulting sterol subsequently eliminates its angular C-19 methyl group (as formic acid) and rearranges to form estrone or estradiol, when the enzyme substrate is androstenedione or testosterone, respectively. Once formed, both estrogens are interconvertible via 17$\beta$-hydroxysteroid dehydrogenase. In young women, the ovaries are the major source of circulating estrogens. After menopause, the production capacity of the ovaries becomes greatly impaired and extraglandular tissues such as fat, liver, and brain, but also breast cancer tissue, assume more significance as sources of estrogen synthesis. Only before menopause are alterations of estrogen levels feedback controlled by pituitary gonadotrophins. As a consequence, and especially in postmenopausal women, inhibition of aromatase should represent an effective way to reduce circulating estrogens when attempting to treat breast cancer.

At present, there are two aromatase inhibitors commercially available: testolactone and AG.

## Testolactone

Testolactone is a testosterone derivative that possesses no androgenic, estrogenic, or progestational activity.[41] Though the agent has been used in treatment of breast cancer since 1960,[42] it was not until 1979 that its activity was found to be due to Type I inhibition of aromatase.[43] In vitro, testolactone behaved as a rather weak, suicide inhibitor:[44] it bound to human placental aromatase with an apparent $K_i$ of 35 $\mu$M, causing a slow time-dependent

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enzyme inactivation with a pseudo-first-order $k_{inact}$ of 0.36 $\times 10^{-3}$/s. Not surprisingly, testolactone has failed to exert convincing therapeutic effects. In a prospective clinical study[46] that involved administration of testolactone (200, 1000, or 2000 mg daily) to about 360 postmenopausal women with stage IV breast cancer, the highest objective response rate noted was 14.6%. This relatively low value (other hormonal agents achieve average response rates between 30% and 50%) has precluded a wider use of testolactone in breast carcinoma.

### Aminoglutethimide

The ability of AG to affect a medical adrenalectomy (vide supra) provided a rationale to treat advanced breast cancer in patients who otherwise would have undergone adrenalectomy.[47] When AG was used as monotherapy in postmenopausal women, it became clear that its effects were not confined to the adrenal cortex. A strong suppression of plasma estradiol and estrone levels, together with a slight enhancement of androstenedione, was noticed, indicating the in vivo capacity of AG to inhibit extragonadal aromatization.[48] Importantly, suppression of estrogen synthesis remained pronounced also when AG was coadministered with the obligatory replacement glucocorticoids. Indeed, clinical experience involving over 1500 postmenopausal women with advanced breast cancer has shown that AG (1000 mg/day) supplemented with corticosteroids (20–40 mg/day) is at least as effective as adrenalectomy or estrogen-receptor blockade by tamoxifen.[49] Overall, aminoglutethimide–corticosteroid treatment achieved an objective response rate of 30–50% with a duration of response of more than 1 year. However, significant side effects occurred in about 40% of patients.[50] These included drowsiness, dizziness, ataxia, and skin rashes. Although most of these phenomena dissipated with time, toxicity seemed to be persistent and pronounced in elderly patients: as a result about one-third of the women older than 65 had to be withdrawn from treatment.[51]

### Recent Aromatase Inhibitors

The search for more potent, selective, and safe aromatase inhibitors has panned out well and led to the development of several novel aromatase inhibitors: suicide substrates, AG analogues, and nonsteroidal inhibitors.

By far the most well-studied suicide substrate and successor to testolactone is 4-hydroxyandrostenedione (4-OHA; Figure 5, **2**). This compound, selected as the most potent from over 200 potential steroidal aromatase inhibitors,[52,53] produced good endocrine effects. In vitro, it interacted with the human placental aromatase with an apparent $K_i$ of 10.2 nM, causing a rapid inactivation with a $k_{inact}$ of 0.41 $\times 10^{-3}$/s.[44,54] In vivo, 4-OHA (2 $\times$ 50 mg/kg per day subcutaneously) almost completely inhibited ovarian and extragonadal estrogen synthesis and reduced the growth of estrogen-dependent mammary tumors.[55,56] These observations have triggered several clinical trials of 4-OHA in postmenopausal women. Weekly intramuscular injections with 500 mg of 4-OHA to 58 unselected breast cancer patients resulted in a 60% suppression of plasma estradiol levels and an overall objective response rate of almost 30%.[57] Daily oral administration of 500 mg of 4-OHA to another 31 patients showed similar biochemical and clinical responses with virtually no signs of side effects.[58] Thus, on the basis of these preliminary data, 4-OHA is clinically more effective and potent than testolactone, while it equals the antitumor activity of AG.

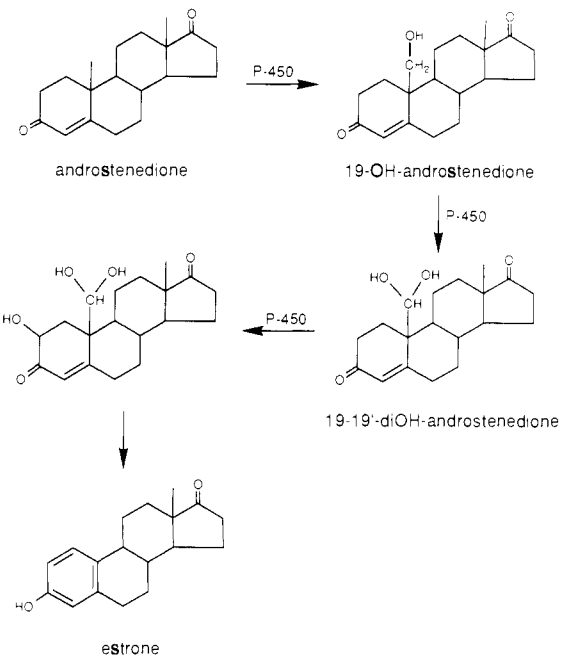


**Figure 5.** Trihydroxylation and aromatization of 4-androstenedione by aromatase.

As mentioned, 4-OHA functions as a suicide substrate: the presence of the 4-hydroxy group, the only structural feature that distinguishes 4-OHA from 4-androstenedione, is responsible for the observed inactivation of aromatase.[44] Many studies have indicated that the design of novel Type I aromatase inhibitors is not confined to derivatization at the C-4 position of androstenedione.[59] Modifications of the C-1, C-6, C-7, C-10, C-16, and C-17 positions, and introduction of double bonds between $C_1$ and $C_2$ or $C_6$ and $C_7$ of $C_{19}$ steroids have been described. The molecular alteration of the C-10 positioned angular methyl (C-19) group has perhaps most often been pursued. As seen in Figure 5, this C-19 group represents an important substrate domain, is subjected to two hydroxylations during the enzymatic reaction, and as such is ideally suited to be modified into a "prereactive" moiety. It is now realized that, dependent on the type of functionalization of the

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C-19 methyl group, either reversible or irreversible Type I inhibitors can be developed. Thus, (19*R*)-10β-thiiranyl- and (19*R*)-10β-oxiranylestr-4-ene-3,17-dione (Figure 4, **3a** and **3b**) proved to be potent, reversible Type I aromatase inhibitors (apparent $K_i$ values of 1 and 7 nM, respectively), which owed part of their activity to coordination of the oxirane oxygen or thiirane sulfur atom with the heme group.[60] In vivo activity of these compounds has not been reported. On the other hand, 10-(2-propynyl)-4-ene-3,17-dione, or MDL 18,962 (Figure 4, **3c**), is the prototype of a mechanism-based enzyme-activated irreversible inhibitor, which binds to placental aromatase with an apparent $K_i$ of 3–4 nM, causing a fast enzyme inactivation (pseudo-first-order $k_{inact} = 1.1 \times 10^{-3}$/s).[59,61] At 1 mg/kg per day orally, **3c** effectively induced regression of mammary tumors in rats and inhibited growth of ovarian carcinoma in athymic mice.[59] Furthermore, the compound inhibited extragonadal estrogen production in baboons with an $ED_{50}$ of 4 mg/kg. Clinical evaluation with **3c** is set for the near future.[59]

Since 1983, several groups have engaged in the synthesis of aromatase inhibitors that are structurally related to AG. Their aim was the same: improve on the enzyme selectivity of AG by designing compounds with enhanced inhibition potency toward aromatase and/or reduced inhibitory effects on desmolase (step 1, Figure 3). Foster et al.[62] found that replacement of the aminophenyl moiety in AG with the more basic 4-pyridyl group to form pyridylaminoglutethimide (Figure 4, 4) had no consequence on antiaromatase activity, but eliminated the inhibitory effects on desmolase. Hartmann and Batzl[63] reported that elongation of the 3-ethyl group in the AG molecule led to increased and more selective inhibition of aromatase. The most active compound, the isopentyl analogue, proved to inhibit aromatase in vitro at least 100 times stronger than AG.

In doses equimolar to 2 mg/kg of AG, many of these 3-alkyl-substituted compounds were superior to the parent drug in suppressing plasma estradiol levels in gonadotrophin-primed rats. Moreover, analogues with medium-chain alkyl substitution ($n$ = 3–5) produced a more pronounced inhibition of testosterone-stimulated tumor growth in ovariectomized rats than AG. Daly et al.[64] synthesized and tested a series of (4-aminophenyl)pyrrolidine-2,5-diones. One of these compounds, 3-(4-aminophenyl)-3-ethylpyrrolidine-2,5-dione (Figure 4, **5**), tested at a dose of 50 mg/kg intraperitoneally, in rats, was equipotent with AG in inhibiting estrogen biosynthesis, but showed little inhibition of cholesterol side-chain cleavage.[65,66]

The initiative for developing nonsteroidal compounds as specific Type II inhibitors of estrogen synthesis was taken only a few years ago. Yet, progress has been considerable as judged by the published in vivo data on the imidazole-containing CGS 16949A (Figure 4, **6**), the indenopyrimidine derivative LY113174 (Figure 4, **7**) and the triazole compound R 76 713 (Figure 4, **8**).

Compound **6** (0.26 mg/kg orally) almost completely reduced ovarian estrogen production in gonadotrophin-primed rats.[67] Furthermore, at oral doses of 1 to 8 mg/kg per day, **6** caused almost complete regression of palpable mammary tumors in rats.[68] Selectivity toward aromatase was indicated by the ability of **6** (4 mg/kg orally) to induce uterine atrophy (due to aromatase inhibition) without inducing adrenal hypertrophy, suggesting there was no interference with adrenal cholesterol side-chain cleavage.[67]

Compound **7** was chemically developed from the agricultural fungicide fenarimol [α-(2-chlorophenyl)-α-(4-chlorophenyl)-5-pyrimidinemethanol].[69] At oral doses of 10–30 mg/kg per day given to rats, this compound strongly inhibited ovarian estrogen production and tumor growth of established mammary carcinoma. No overt signs of side effects were observed, although relative liver weights were increased as compared with controls.

Compound **8** proved to be a potent, long-acting, and selective aromatase inhibitor in rats.[70] An oral dose as low as 0.1 mg/kg lowered plasma estradiol levels of gonadotrophin-primed animals by more than 90% for at least 8 h. In contrast, dosing with **8** (20 mg/kg orally) had no effect on plasma levels of testicular or adrenal steroids in pituitary hormone injected rats. Furthermore, no estrogen or androgen agonistic/antagonistic effects were seen.

Judging from the length of this section, the development of novel aromatase inhibitors has been much more prolific than that of 17-hydroxylase/17,20-lyase blockers. Is aromatase a relatively easy P-450 system to inhibit? It appears so. There is now compelling evidence[71] that the aromatization of $C_{19}$ steroids in humans is catalyzed by a single enzyme. In other words, one active site is responsible for carrying out the three sequential hydroxylation reactions at different positions on the steroid molecule. In order to accomplish this versatility, the aromatase must be able to shift the substrate around its heme site. This shifting would inevitably lead to a reduced selectivity of the substrate binding domain and to a higher probability to interact with inhibitory compounds.

## Other Carcinomas

Prostate and breast carcinomas are by far the major sex hormone dependent malignancies that afflict mankind. Each year, in the United States alone, about 100 000 men and 130 000 women develop prostate cancer or invasive breast cancer, respectively.[72] Other neoplasms, such as endometrial, hepatocellular, and adrenal carcinomas, could be considered for treatment with P-450 inhibitors. There is indeed strong evidence that endogenous steroids are also involved in the establishment and growth of endometrial carcinoma.[73] Excess of estrogen production and/or absence of cyclic changes of progesterone levels are typically associated with this disorder. Already since 1960, prog-

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estational compounds such as medroxyprogesterone, megestrol, and 17α-hydroxyprogesterone have been successfully used in the treatment of this disease with objective remission rate of 30–40%.[73,74] That success may explain why the therapeutic approach of aromatase inhibition has seldom been explored. However, from the only double blind, randomized trial performed, it was concluded that a combination of AG (1000 mg/day) and hydrocortisone (40 mg/day) was as effective as megestrol acetate (160 mg/day) in causing regression of metastatic endometrial carcinoma.[41]

Hepatocellular carcinoma is also often associated with a derangement of sex hormone metabolism occurring in patients with underlying cirrhosis.[75] In particular, the elevated plasma concentrations of estrogens are thought to play a malafide role in hepatocarcinogenesis of the cirrhotic liver.[76] If correct, reduction of circulating levels of estrogens by aromatase inhibition could represent a valuable strategy to prevent the development or to slow down the growth of this type of liver carcinoma. As yet, no attempts in that direction have been reported.

Adrenal carcinoma is a special case: it is not steroid-dependent sensu proprio. Rather, this disease is characterized by hypersecretion of steroid hormones, in most cases, of cortisol. The resulting hypercortisolism produces a pathological condition called Cushing's syndrome with hypertension, electrolyte balance disturbance, muscular weakness, fragile blood vessels, and altered fat metabolism as the most prominent pathophysiological features.[77] The condition is clinically serious and, if left untreated, has a poor prognosis. Both HD ketoconazole[78] and AG[29] have been used for palliative treatment of this disorder. Although these drugs markedly attenuated Cushing's symptoms, they had no consistent effect on the cancer development itself.

The contribution of sex hormones in the development of tumors of other organs is less clear. Even for cancers of the ovaries and testes, there is limited evidence that manipulation of steroid hormone levels could be of benefit to patients.[73]

## Metastasis

Nearly all malignant neoplasms metastasize. The process of metastasis involves a number of sequential steps:[79] shedding of tumor cells from the primary neoplastic tissue, penetration through the blood vessel wall to enter the circulation, transfer via the vascular system, arrest in the microvasculature or adhesion to the endothelium, extravasation through the vessel wall and lodging in the adjacent tissue. In theory, the survival rate of blood-borne tumor cells is low, exposed as they are to immune attack and mechanical trauma during their passage through the vascular system. However, the usual propensity to metastasize tells another story. Tumor cells seem to have devised successful ways to protect themselves. One such possible way has been brought forward by Honn and coworkers.[80,81] They argued that, once in circulation, malignant cells disrupt the intravascular balance between the prostanoids prostacyclin and thromboxane ($TxA_2$), resulting in platelet aggregation. By combining with the platelet microaggregates, tumor cells would be shielded against damaging effects of blood-borne immune cells or shear forces. In addition, platelet thrombi could adhere tumor cells to the vascular wall, whereas tumor growth might be enhanced by platelet-derived mitogenic factors.

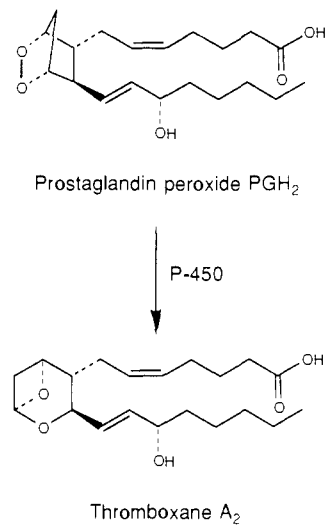


**Figure 6.** Molecular rearrangement of $PGH_2$ to thromboxane $A_2$ by thromboxane synthetase.

If this line of reasoning is correct, then agents that augment prostacyclin biosynthesis or activity or block $TxA_2$ production or action should possess antimetastatic properties. In this respect, inhibition of $TxA_2$ synthetase could be a valuable approach. $TxA_2$ synthetase is a P-450-dependent enzyme,[82] capable of activating one of the endoperoxide bonds of its substrate $PGH_2$ and of stereospecifically rearranging $PGH_2$ to form $TxA_2$ (Figure 6).[83] To perform this rearrangement, the synthetase operates in its oxidized ferric state and neither oxygen nor electrons are required.[82] However, interaction of carbon monoxide or Type II inhibitors with the enzyme produces the expected spectral changes. The basic structural requirements for Type II inhibition are well established:[84] a 1-imidazolyl or a 3-pyridyl moiety at one end of the molecule and a carboxyl acid group at the other. Optimal activity is obtained when the distance between the carboxyl group and the free nitrogen atom of the imidazole or pyridine ring varies between 8.5 and 10 Å (a similar distance separates the COOH group and the endoperoxide moiety in the $PGH_2$ molecule). To date, numerous orally active and potent $TxA_2$ synthetase inhibitors have been synthesized, but data on their antimetastatic activity in experimental animals have been scarce and conflicting.

Honn et al. reported that OKY 1553 (Figure 4, **9**) significantly suppressed metastasis from B16a and 3LL tumors to the lungs in mice.[85] In contrast, Vicenzi et al., using a highly metastatic variant of a fibrosarcoma, failed to show antimetastatic effects with dazmegrel (Figure 4,

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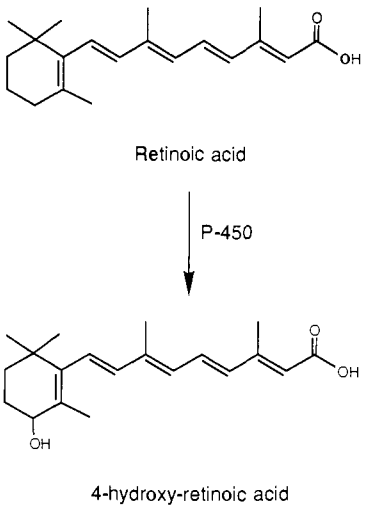


**Figure 7.** Hydroxylation of retinoic acid.

10), although this compound strongly depressed $TxA_2$ generation by the tumor cells.[86] Similarly, Stamford and co-workers found dazmegrel inactive on metastasis of NC carcinoma to lungs in mice.[87] Apparently, mere reduction of thromboxane levels does not result in suppression of metastasis. However, blocking $TxA_2$ synthesis inevitably leads to preservation of the thromboxane precursors $PGH_2$ and $PGG_2$. These endoperoxides are able to interact with $TxA_2$ receptor sites on platelets and to induce their aggregation despite synthetase inhibition. Experiments with mixtures of a $TxA_2$ synthetase inhibitor and a $TxA_2$ receptor blocker or with compounds that combine antisynthetase and antireceptor activities would indeed be very instructive to establish unequivocally the contribution of this particular P-450 inhibition in the process of metastasis.

## Epithelial Differentiation

Cancer is essentially a derailment of cellular differentiation, mostly occurring in epithelial tissues. The principal endogenous molecule responsible for controlling differentiation and growth of epithelia is retinoic acid (RA), a physiological derivative of vitamin A. Indeed, several experiments in laboratory animals have demonstrated that RA inhibited the induction and caused the disappearance of tumors.[88] In spite of these positive results, the effects of RA on established human epithelial cancer were rather meager.[89] Why this clinical failure? One plausible reason could be the high rate of metabolism and degradation of RA resulting in drastic impairment of its biological efficacy.[90,91] The most important route of RA biodegradation consists of the P-450-dependent hydroxylation at the C-4 position of the cyclohexenyl ring to form 4-hydroxy-RA (Figure 7).[92-95] Logically, inhibition of this 4-hydroxylation reaction would result in delay of RA breakdown, more sustained/higher tissue concentrations of RA and improved control of neoplastic differentiation and growth. Thus, P-450 inhibitors endowed with the capacity to inhibit the enzymatic conversion of RA would enhance the endogenous tissue levels of RA and exhibit antitumor activity. This reasoning is not merely speculative: we recently demonstrated the feasibility to design P-450 inhibitors that could prolong the biological half-live of exogenously administered RA to rats.[96]

## Concluding Remarks

So, is there a case for P-450 inhibitors in cancer treatment? Let us admit that this question comes too early. Up till now only three P-450 inhibitors have undergone thorough clinical evaluation as anticancer agents: ketoconazole in prostate carcinoma, AG and testolactone in breast cancer. Ketoconazole and AG were introduced into oncologic practice because of serendipitously found side effects. This statement is not meant to belittle serendipity, but merely to indicate that neither ketoconazole nor AG were conceived as antineoplastic agents. Ketoconazole, an effective and safe antifungal drug when taken at a dose of 200 mg/day, had to be "overdosed" to generate consistent effects on the steroidogenic P-450 system in humans. The resulting HD ketoconazole (400 mg every 8 h), though of value in treatment of prostatic cancer, was therefore not further developed for this indication. AG, introduced as an anticonvulsant, did not match the expectations and was subsequently developed as a drug capable of exerting medical adrenalectomy and inhibiting aromatase and, hence, primarily usable for treatment of advanced breast cancer. No doubt, AG exhibits antitumor effects. However, its action is too often accompanied by lack of selectivity and moderate tolerability. By contrast, testolactone is very well tolerated, but unfortunately its antitumor activity is rather modest.

On a positive note, the clinical experience gained with these three drugs has indicated the clinical potential of more potent and selective P-450 inhibitors of human steroidogenesis. The first, careful steps in that direction are now being taken with (pre)clinical testing of novel aromatase and 17-hydroxylase/17,20-lyase inhibitors. Time will tell whether the P-450 approach to cancer therapy represents a valuable asset for oncologists.

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**Registry No.** Cytochrome $P_{450}$, 9035-51-2.

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