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Natural Modulators of Estrogen Biosynthesis and Function as Chemopreventive Agents

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There is clearly a need for novel breast cancer chemopreventive agents with enhanced potency and specificity with little or no side effects. To this end, several new chemical moieties have been synthesized or isolated from natural sources. In this review, we have described some agents currently in use or under development for treatment or prevention of breast cancer, as well as our own strategies for the discovery of natural product modulators of estrogen biosynthesis and function. In particular, bioassay-guided fractionation of active plant extracts is a unique method for identifying agents with novel mechanisms of action, some of which should be useful for prevention of human cancer. Further, with the advent of combinatorial chemistry and high throughput screening, even greater progress may now be expected with natural product leads.

Key words: Antiestrogens, Aromatase inhibition, Sulfatase inhibition, Breast cancer, Natural products

INTRODUCTION

At the present time, breast cancer accounts for up to one third of all new cases of cancer in North America, representing the most common neoplastic disease experienced by women (Landis *et al.*, 1999). Although various factors may influence the outcome of the disease, the importance of unopposed exposure to elevated levels of estrogens (Henderson *et al.*, 1988) has been indicated for a variety of female cancers (Greenwald *et al.*, 1971; Nissen and Kent, 1975; Herbst, 1981). Overall, our current understanding of the carcinogenicity of estrogens is based on clinical observations associating a greater risk of endometrial hyperplasia and neoplasia with estrogen supplementation (Shaw, 1987; Chilvers *et al.*, 1987) and experimental evidence (Yan and Roy, 1995; Tsutsui and Barrett, 1997). Estrogens can induce cancer by either stimulating cell proliferation via the estrogen receptor (ER) pathway (Adlercreutz *et al.*, 1994; Nandi *et al.*, 1995), or by direct genotoxic effects such as an increase in mutation rates (Liehr *et al.*, 1986; Bolton *et al.*, 2000; Chen, *et al.*, 2000; Pisha, *et al.*, 2001), or affecting the DNA repair system leading to accumulation of lesions in the genome that act as precursors of estrogen-induced tumorigenesis (Yan and Roy, 1995).

Relevant to the above effects, the role of estrogens in the growth, differentiation, and function of many reproductive tissues has been widely studied. In addition, estrogen exerts important actions in tissues outside of the reproductive system, including bone, brain, and liver, and the cardiovascular system (Katzenellenbogen, 1996; Katzenellenbogen *et al.*, 1997). Most of the actions of the estrogens appear to be exerted via the ER, an intracellular protein belonging to a family of hormone-activated transcription factors that can initiate or enhance the transcription of genes containing specific hormone response elements (MacGregor and Jordan, 1998). However, the discovery of a second estrogen receptor, ER-beta, which can have effects opposite to those of the well-known 'original' estrogen receptor (now called ER-alpha), challenges this simplistic view (Kuiper *et al.*, 1996). Although estradiol (E_2) binds with equal affinity to both subtypes, certain ligands, such as phytoestrogens, exhibit preferential binding to ER-beta. These factors are clearly relevant for drug development, but since reviews describing mechanistic

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aspects of signal transduction mediated by ERs have recently appeared (Curtis Hewitt *et al.*, 2000; Katzenellenbogen *et al.*, 2000), we will focus on other aspects of estrogenicity as therapeutic targets. These will include selective estrogen receptors (SERMs) and modulators of aromatase and estrogen sulfatase.

Agents for the Treatment and Prevention of Breast Cancer

Selective estrogen receptor modulators and phytoestrogens

It was not until 1990 that the idea of selective estrogen receptor modulation was conceptualized. Jordan and colleagues, realizing that the effect of tamoxifen was not restricted to the breast and uterus, but also applied to other ER-positive target tissues such as bone and heart, proposed multiple applications for antiestrogens (Lerner and Jordan, 1990). Tamoxifen and its derivatives are partial estrogen agonists in the uterus that also inhibit 7,12-dimethylbenz[a]anthracene (DMBA)-induced rat mammary tumor growth and the *in vitro* growth of ER-positive MCF-7 breast cancer cells (Jordan, 1976; MacGregor and Jordan, 1998). Tamoxifen belongs to the first generation of selective estrogen receptor modulators. Raloxifene, originally developed for the treatment of osteoporosis and now approved for its prevention, has so far proved to be a superior SERM in preclinical studies (Fuqua *et al.*, 2001). This agent possesses antiestrogenic properties in breast and uterus, and estrogenic effects in bone and heart, as seen by a decreased incidence of osteoporosis and levels of low-density lipoproteins (LDL). Based on these results, several clinical trials have either been initiated or completed, such as Raloxifene Use for The Heart (RUTH) and Multiple Outcomes of Raloxifene Evaluation (MORE) (Fuqua *et al.*, 2001). SERMs bind to the ER and display differential estrogenic/antiestrogenic effects in a tissue-, promoter-, and species-specific fashion (Katzenellenbogen *et al.*, 2000). The SERM-ER complexes are believed to have unique structures that influence their activity in different tissues. Moreover, binding of co-regulatory proteins are thought to influence the activity of SERMs in a tissue-specific manner. Several SERMs are marketed or in clinical development, including triphenylethylenes such as tamoxifen and its derivatives, chromans such as levormeloxifene, benzothiophenes such as raloxifene and LY353381, and naphthalenes (Dardes and Jordan, 2000).

Phytoestrogens have been long studied for their effects on estrogen biosynthesis and function. Prompted by reproductive disturbances in sheep grazing clover pastures, epidemiological and pharmacological studies have established the estrogen modulatory effects of phytoestrogens (Bennetts *et al.*, 1946). In human beings, soybeans and flaxseed are the most common source of phytoestrogens of which isoflavones represent a major constituent (Brzezinski and Debi, 1999). Asians, whose traditional oriental diet is rich is phytoestrogens, have lower incidence rates of breast, prostate and colon cancer, compared to their Western counterparts (Adlercreutz, 1995; Rose *et al.*, 1986). In addition, a few studies indicate that Japanese women experience reduced post-menopausal symptoms such as hot flashes (Adlercreutz *et al.*, 1992). A large body of evidence also supports the notion that phytoestrogens reduce the risk of heart disease in postmenopausal women (Lissin and Cooke, 2000). Soy-based diets have been shown to decrease the levels of cholesterol, LDL and triglycerides, and soy extracts have a direct stimulatory effect on bone formation in cultured osteoblastic cells (Carusi, 2000; van der Schouw *et al.*, 2000). In addition, preventive effects of isoflavones on bone loss due to suppression of bone turnover have been established with *in vivo* studies (Uesugi *et al.*, 2001). These interesting observations suggest that phytoestrogens alter a common pathway in these tissues, and the most likely possibility is ER signaling.

However, experimental efforts to define and validate these epidemiological findings have been relatively unsuccessful. This can be attributed to variations in experimental models, differences in dose regimens and test agents (e.g., natural soy versus soy dietary supplements), that can result in conflicting data. For example, some studies have indicated that phytoestrogens are estrogenic in mammary cancer cell lines (Dees *et al.*, 1997; Hsieh *et al.*, 1998) and can cause an increase in the uterine weights of rats, while others have demonstrated reduced tumor incidence or multiplicity in animal models (Fournier *et al.*, 1998). Thus far, genistein is the most promising chemopreventive isoflavone to have been studied. This substance binds to ER, promotes proliferation of reproductive organs in animals (Whitten *et al.*, 1992), induces pS2 protein expression (Wang *et al.*, 1996), and stimulates growth in estrogen-dependent human breast cancer cells (Makela *et al.*, 1994; Wang and Kurzer, 1997). Other studies have shown that genistein exhibits simultaneous estrogen agonist as well as cell growth-inhibitory actions over a physiologically relevant concentration range (Le Bail *et al.*, 1998). While one school of thought suggests that genistein is estrogenic in a low-estrogen environment and antiestrogenic in high estrogen environment, it has also been suggested that the time of phytoestrogen exposure plays a major role in protecting against or promoting cancer. In a study by Lamartiniere *et al.* (1998), pharmacologic doses of genistein given to immature rats enhanced mammary gland differentiation, and this resulted in a situation wherein the gland was significantly less proliferative and less susceptible to mammary cancer. It has also been shown that rats treated prepubertally with genistein had a reduced incidence of DMBA-

induced tumors, together with an increased latency period, whereas rats treated with genistein after 35 days of age had less protection against cancer risk (Barnes, 1997). These studies support the work of Nandi and colleagues, who have hypothesized that mimicking pregnancy by short-term specific hormonal exposure protects against breast cancer in nulliparous rats (Guzman *et al.*, 1999).

Another interesting observation is that phytoestrogens do not reduce the incidence of established tumors. To the contrary, tumor cell proliferation may be enhanced. For example, Hsieh *et al.* (1998) have shown that genistein stimulates the growth of mammary gland and MCF-7 cell tumors in ovariectomized athymic mice, and McMichael-Phillips *et al.* (1998) reported that daily consumption of soy product for two weeks increased DNA synthesis in breast tissue obtained through biopsies of premenopausal women. However, cell proliferation is not necessarily an indication of tumor prognosis, and the increase in cell number is a compensatory response to apoptosis caused by chemopreventive agents (Moorghen *et al.*, 1998; Zhou *et al.*, 1998). On the other hand, when genistein has been administered concomitantly with estradiol or at high levels, cell proliferation was decreased (Wang *et al.*, 1996; Shao *et al.*, 1998).

Another mechanism by which phytoestrogens have been proposed to influence carcinogenesis is via effects on tyrosine kinase activity (Akiyama *et al.*, 1987). Genistein has been shown to inhibit tyrosine phosphorylation of proteins in signal transduction pathways such as platelet-derived growth factor receptor (PGFR), epidermal growth factor receptor (EGFR), and insulin receptor substrate I (Davidai *et al.*, 1992; Kawase *et al.*, 1995). However, the concentrations required for these effects have generally been higher than levels reported in human plasma. In sum, phytoestrogens may demonstrate either estrogenic or and antiestrogenic properties, depending on the model employed (*in vitro* or *in vivo*), dose, and time of administration.

It appears that a similar profile can be observed with raloxifene. This compound is not effective in the treatment of breast cancer, but it can effectively suppress carcinogenesis as well as mediate multiple beneficial effects in post-menopausal women. There are not sufficient data to recommend the use of phytoestrogens in place of traditional estrogen replacement therapy (ERT) or as SERMs. Clinical trials are required with human beings and, if the results correlate with certain *in vitro* and animal studies, specific recommendations could be made. In the meanwhile, bearing in mind the long-term consumption of soy products, it is somewhat ironic that no definitive conclusions can be drawn in regard to health benefits.

One of the cell-based assays we have included in our chemoprevention program involves screening the anti-estrogenic potential of plant extracts. The Ishikawa cell line was derived from a well-differentiated endometrial adenocarcinoma of glandular epithelial cells (Nishida *et al.*, 1985). Previous endometrial cell lines were derived from moderately or poorly differentiated adenocarcinomas that did not respond to estrogen or progesterone (Gal *et al.*, 1982; Lindahl *et al.*, 1984), but Ishikawa cells respond to estrogens at concentrations approximating physiological levels (Holinka *et al.*, 1986). A predominant effect of exposing Ishikawa cells to estrogens is rapid proliferation (Holinka *et al.*, 1989). The levels of basic fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF) are greatly increased by estrogen exposure (Fujimoto *et al.*, 1997; Fujimoto *et al.*, 1999). 17β-Estradiol (E_2) also increases DNA polymerase α activity, ODC activity, c-fos expression and PKC activity in Ishikawa cells (Gravinis and Gurpide, 1986; Holinka *et al.*, 1986; Fujimoto *et al.*, 1996). These activities are estrogen-dependent as demonstrated by inhibition of the response when E_2 was co-incubation with an antiestrogen (Gravinis and Gurpide, 1986; Holinka *et al.*, 1986; Fujimoto *et al.*, 1996, 1997; Lessey *et al.*, 1996). Determination of specific binding with tritium-labeled E_2 and progesterone indicated the presence of ER and progesterone receptor (PR), which was confirmed by immunocytochemical analysis (Hata and Kuramoto, 1992).

One useful response mediated by Ishikawa cells on exposure to estrogens is the induction of alkaline phosphatase (AP) (Albert *et al.*, 1990). A four-day incubation with 10 nM E_2 increased the level of AP activity 5-8-fold as compared to control cells; the stimulation was dose-dependent with maximal induction at 1-100 nM (Holinka *et al.*, 1986). The induction is specific for estrogenic compounds. Estriol (10 nM) induced AP activity to a level comparable to that induced by E_2. Estrone (10 nM) and 17a-E_2 (10 nM) stimulated enzyme induction about half as much as E_2 (10 nM). Prednisolone (1 μM), dexamethasone (1 μM), testosterone (1 μM), prolactin (250 ng/ml), EGF (100 ng/ml) and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (0.32 M) had no effect on the basal activity of AP (Holinka *et al.*, 1986). The inability of testosterone to induce enzymatic activity indicates a low level of aromatase in the Ishikawa cells, which may not facilitate the conversion of testosterone to estrogenic derivatives (Yamamoto *et al.*, 1993).

Another essential feature of AP induction in Ishikawa cells is adaptability to a 96-well plate format (Littlefield *et al.*, 1990; Pisha and Pezzuto, 1997). In the range of 10^{-7} -10^{-12} M, E_2 demonstrated dose-dependent induction of AP on microtiter plates; induction was measurable at 10^{-12} M, with a maximum at 10^{-9} M. Several natural and synthetic estrogens also were capable of inducing enzymatic activity on the 96-well plates, and the specificity of the induction was not lost in the microtiter adaptation, since various corticoids, progestins and androgens were incapable of stimulating activity in the 96-well plates, similar to data observed with the larger-scale experiments

(Littlefield *et al.*, 1990). Further, the addition of antiestrogens inhibit estrogen-dependent AP induction in both large-scale and microtiter plate experiments (Anzai *et al.*, 1989; Littlefield *et al.*, 1990). Antiestrogenic effects demonstrated with EM-800 (Simard *et al.*, 1997) and tamoxofen (Pisha and Pezzuto, 1997). Similarly, plant derived phytoestrogens such as genistein and apigenin have been shown to have estrogenic activity (Pisha and Pezzuto, 1997).

Other *in vitro* assays for determining estrogenic/antiestrogenic activity include ER binding assays, the 'E-screen', ER transformed yeast and, more recently, an assay has been developed for identification of SERMs by monitoring gene expression fingerprints. The ER-binding assays do not reveal the potential of an agent to activate or inhibit transcription. Thus, cell-based assays are normally utilized to confirm activity. The 'E-screen' exploits estrogen-dependent growth enhancement, and induction of PR and pS2 in MCF-7 cells, and the ability of antiestrogens to suppress this effect (Soto *et al.*, 1995). In another model for determining estrogenic activity, a yeast, *Saccharomyces cerevisiae*, was transfected with the human ER complimentary DNA and this resulting organism was further transfected with a plasmid containing a specific promoter region upstream from the coding sequence of the *Escherichia coli* β-galactosidase gene (Metzger *et al.*, 1988; Pham *et al.*, 1992). The doubly transfected yeast demonstrated dose-dependent induction of β-galactosidase activity in the presence of E_2. Finally, Zajchowski *et al.* (2000) have recently reported an assay to classify estrogen agonists and antagonists based on their gene expression fingerprints (GEF). The resulting data show a good correlation with *in vivo* systems suggesting that GEF-based screens could be useful in predicting the *in vivo* pharmacological profiles of test compounds.

During the course of our work, we have identified resveratrol (Fig. 1), a chemopreventive phytoalexin (Jang *et al.*, 1997), as potentially beneficial for hormone-dependent cancer (Bhat and Pezzuto, 2001; Bhat *et al.*, 2001). In Ishikawa cells, resveratrol mediates antiestrogenic effects by a novel mechanism that involves selective down-regulation of ER-alpha, but not ER-beta, manifested as suppression of AP and estrogen response element (ERE)-luciferase activities, as well as PR and α1 integrin expression. In addition, resveratrol exhibits cytostatic activity in these cells by prolonging S-phase and selectively modulating cyclins A and E, and cdk2 expression (Bhat and Pezzuto, 2001). With mammary tissues, resveratrol mediates mixed estrogenic/antiestrogenic effects. In the absence of E_2, resveratrol weakly induced ER-dependent transcriptional events in some mammary tumor cell lines, but down-regulation was invariably observed when the agent was co-administered with E_2. In mouse mammary glands grown in culture, resveratrol inhibited the formation of DMBA-induced, E_2-promoted, atypical

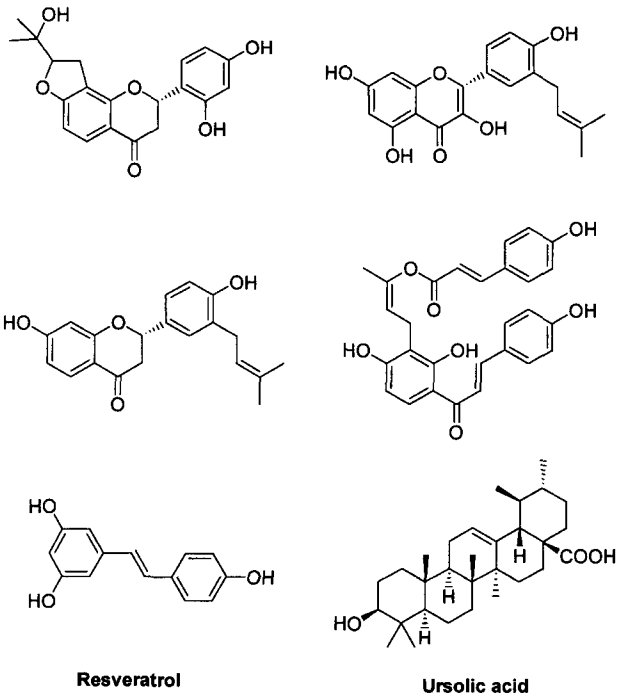

Fig. 1. Structures of novel modulators of estrogen biosynthesis and function.

ductal hyperplasia. Further, resveratrol inhibited the early stages of *N*-methyl-*N*-nitrosourea (NMU)-induced mammary carcinogenesis when administered to female Sprague-Dawley rats (Bhat *et al.*, 2001). In conjunction with previous work reported in the literature (such as attenuation of bone loss, and cardiovascular benefits of resveratrol), these studies support the suggestion that resveratrol is a novel SERM that may be useful for the chemoprevention of breast cancer.

Aromatase inhibitors

As noted above, epidemiological and experimental evidence strongly supports a role for estrogens in the development and growth of breast cancer (Vogel, 1996; Reddy, 1998), and a role for estrogens in prostate neoplasia has also been postulated (Henderson *et al.*, 1987). Therefore, a chemotherapeutic or chemopreventive strategy for breast cancer and prostate cancer control is to decrease estrogen production (Kelloff *et al.*, 1998). The biosynthesis of estrogens from androgens is catalyzed by aromatase, an enzyme complex consisting of a cytochrome P_{450} (CYP_{450}) hemoprotein and a flavoprotein, NADPH-CYP_{450} reductase (Brueggemeier, 1990). This complex converts C_{19} steroids into C_{18} steroids containing an aromatic A ring, which is a key step in the synthesis of estrogens. Aromatase activity was first reported in the microsomal membrane fraction from human term placenta in 1959 (Canick and Ryan, 1976), but it is now known to occur

in other tissues throughout the body. Since this enzyme catalyzes the final, rate-limiting step in estrogen biosynthesis, inhibitors should be effective for the treatment of breast and prostate cancers (Cole and Robinson, 1990). The use of selective and potent aromatase inhibitors is of clinical interest.

Several classes of aromatase inhibitors, including substrate androstenedione derivatives, nonsteroidal aminoglutethimides and its analogues, imidazoles, and triazoles, have been developed over the past 20 years as potential therapeutic agents (Kelloff *et al.*, 1998). Aminoglutethimide (AG) was the first aromatase inhibitor to be used clinically. It was effective in reducing NMU-induced tumor incidence in Sprague-Dawley rats when administered at 400 mg/kg diet (Moon *et al.*, 1994). However, due to the non-selective action of this compound, its structural similarity to phenobarbital and associated CNS effects, and lack of potency relative to triazole aromatase inhibitors, usage is not common (Kelloff *et al.*, 1998). Rogletimide, an AG analog that is more specific but less potent than AG, was effective in reducing testosterone-induced increases in tumor size (Yamamoto *et al.*, 1991). Several triazole, non-steroidal compounds have been effective aromatase inhibitors. For example, vorozole is one of the most potent and specific aromatase inhibitors. At p.o. doses of 5 mg/kg, vorozole decreased the % tumor incidence from 100 to 10, and the multiplicity from 5 to 0.1 tumors/animal, in Sprague-Dawley rats treated with NMU (Lubet *et al.*, 1994). However, one major drawback of this drug is that it mediates androgenic activity and, as a result, the animals appeared bulky and heavily muscled (De Coster *et al.*, 1992). Among the steroidal inhibitors, exemustane (administered s.c.) was shown to be effective in causing DMBA-induced tumor regression and prevented the formation of new tumors (Zaccheo *et al.*, 1991). This drug, however, also mediates androgenic effects.

Vorozole and liarozole, alone and in combination with retinoids, are currently being evaluated in clinical trials for the chemoprevention of breast and prostate cancer (Kelloff *et al.*, 1998). However, there are neither enough leads nor sufficient data with existing aromatase inhibitors that can support their use as chemopreventives. Plant-derived aromatase inhibitors may be helpful, especially if there is experience with human consumption. Based on these considerations, we have searched for aromatase inhibitors as part of our chemoprevention program. Model systems used for testing the efficacy of aromatase inhibitors have been reviewed by Goss and Gwyn (1994). Cell-free studies can be carried out with microsomal aromatase preparations from human placenta or pregnant mare serum gonadotropin (PMSG)-stimulated rat ovaries. Tritiated androgens are added to these preparations in the presence or absence of a potential inhibitor and a NADPH-generating system, and the amount of tritiated water released is proportional to the rate of estrogen synthesis.

Cell culture systems for screening aromatase inhibitors include the hormone-dependent human breast cancer cell line, MCF-7 (Kitawaki *et al.*, 1993), and human genital skin fibroblasts (Evans *et al.*, 1995). Also, Zhou *et al.* (1990) and Yue *et al.* (1994) have constructed aromatase expressing transfected mammalian cell lines that may be useful tools for aromatase inhibitor screening.

In vivo models for screening aromatase inhibitors include isolation of ovarian microsomes from pregnant mare serum gonadotropin PMSG-primed female rats treated with the aromatase inhibitor, followed by measurement of radiolabeled water released after *in vitro* incubation with tritiated androgens (Di Salle *et al.*, 1990). In addition, serum estrogen levels of animals treated with aromatase inhibitors can be determined using RIAs (Zaccheo *et al.*, 1991). Carcinogens such as NMU and DMBA both induce multiple mammary tumors in Sprague-Dawley rats within 6 weeks of administration, of which 80-90% are hormone-dependent. The ability of aromatase inhibitors to suppress the formation of tumors has been widely studied. Athymic mice co-inoculated with MCF-7 cells and matrigel, a basement membrane preparation, has also been used for determining the potency of aromatase inhibitors.

We have employed human placental microsomes as a primary source of aromatase. This assay is relatively quick and utilizes 1β, 2β-[^{3}H]4-androsten-3,17-dione labeled with tritium in positions 1 and 2 as the substrate. Product formation occurs in approximately three minutes and the released tritated water in the aqueous phase (separated by addition of chloroform to the reaction mixture) is measured by scintillation counting.

Broussonetia papyrifera is an edible deciduous tree, parts of which have been used for treatment of impotency and ophthalmic disorders (Ko *et al.*, 1997). In our search for edible aromatase inhibitors, we found an extract of *B. papyrifera* to have significant activity (0.4 μg/ml). Bioassay-guided fractionation of a *B. papyrifera* extract using the *in vitro* aromatase inhibition assay led to the isolation of five novel and ten known active compounds (Lee *et al.*, 2001). They included coumarins, benzofurans, biphenylpropanes and various types of flavonoids. Out of a series of 42 compounds tested, compounds **1** (2*S*-2',4'-dihydroxy-2''-(1-hydroxy-1-methylethyl)-dihydrofuro[2,3-*h*]flavanone; IC_{50} 0.11 μM) and **2** (5,7,4'-trihydroxy-3'-prenylflavonol; IC_{50} 0.13 μM) (Fig. 1) were the most potent, exhibiting approximately 60-fold greater potency than aminoglutethimide. Compounds **3** (2*S*-abyssinone II; IC_{50} 0.37 μM) and **4** (3'-[γ-hydroxymethyl-(*E*)-γ-methylallyl]-2,4,2',4'-tetrahydroxychalcone 11-*O*-coumarate; IC_{50} 0.52 μM) were also significantly active (Fig. 1). Initially, some of the compounds were tested for binding to ER-alpha or ER-beta. Interestingly, none of the aromatase active compounds showed significant binding to the

either of these receptors. This is important since potent estrogenic effects could be mediated by aromatase inhibitors, and such an effect could outweigh the potential benefits of inhibiting aromatase. Compound **1** also was effective in inhibiting (50%) the formation of alveolar lesions in a mouse mammary organ culture model when tested at 100 ng/ml. In addition to inhibition of aromatase, flavonoids and other non-steroidal aromatase inhibitors can affect non-specifically the activity of phase I and II enzymes (Le Bail *et al.*, 1998). As a preliminary study, we evaluated the potential of some of the flavonoids to induce quinone reductase, a phase II enzyme involved in detoxification mechanisms. Employing the murine hepatoma 1c1c7 cell culture system (Song *et al.*, 2000), no significant (IC_{50} >40 μg/mL) induction of the enzyme was observed with any of the agents tested (data not shown).

Thus, inhibition of aromatase was achieved at physiologically relevant concentrations (100-1000 nM) with dietary flavonoids. Of additional interest, the fruit of this plant has been consumed in China for the treatment of various disorders. Considering the NCI strategy for identifying chemopreventive aromatase inhibitors (Kelloff *et al.*, 1998), and bearing in mind that these agents may be relatively non-toxic, further experiments have been instituted that involve large-scale synthesis and testing of compound **2** in animal models of mammary carcinogenesis. This work is supported by the RAPID program of the NCI. To our best knowledge, these are the most potent aromatase inhibitors derived from nature, and is logical to suggest significant potential for development as chemopreventive agents.

Estrone sulfatase inhibitors

Many breast tumors are hormone-dependent, and considerable research effort has been directed towards the development of novel therapies to block the action or synthesis of estrogens. One such strategy, as detailed above, is the inhibition of aromatase. Much of the estrone synthesized by the aromatase, however, is converted to estrone sulfate by the enzyme estrone sulfotransferase (Hobkirk, 1985). Plasma and tissue concentrations of estrone sulfates are much higher than those of unconjugated estrogens. Furthermore, the half-life of estrone sulfate is 10-20-times greater than that of estrone or E_2 (Ruder *et al.*, 1972). It has therefore been suggested that estrone sulfate may act as a reservoir for the formation of estrogens by the action of estrone sulfatase, an enzyme which hydrolyzes estrone sulfate to estrone (Pasqualini, 1996). The activity of estrone sulfatase in breast tumors is considerably greater than aromatase activity, and using the appropriate substrate concentrations, formation of estrone via the sulfatase pathway was found to account for at least 10-times as much estrone as that formed via the aromatase route (Santner *et al.*, 1984). Support for the concept of estrone sulfate acting as a reservoir for the formation of estrone and E_2 was provided from studies in which peripheral aromatase activity was completely inhibited by the treatment of breast cancer patients with aromatase inhibitors (Reed *et al.*, 1990). It was found that despite the effective block of aromatase activity, plasma estrone and E_2 concentrations decreased only by 50-60 %, and plasma estrone sulfate concentrations also remained relatively high. The most likely source of the estrone and E_2 that was detectable in the plasma of women treated with aromatase inhibitors was probably estrone sulfate, which is hydrolyzed by estrone sulfatase.

Therefore, due to the potential importance of estrone sulfate for the formation of estrone and E_2 in post-menopausal women with breast cancer, a series of estrone sulfatase inhibitors was developed for use, either alone or in association with an aromatase inhibitor, as potential therapeutic agents (Duncan *et al.*, 1993; Howarth *et al.*, 1994). Of all the estrone sulfatase inhibitors reported in the literature, estrone-3-*O*-sulfamate is the most potent inhibitor with an IC_{50} value of ~80 nM in human placental microsomes, although a few tricyclic coumarin sulfamates have been recently been identified as potent agents (Purohit *et al.*, 1995; Purohit *et al.*, 2001). However, estrone-3-*O*-sulfamate is a potent estrogen and may not be ideal in a chemopreventive setting. Chu *et al.* (1999) report the development of (*E*)- and (*Z*)-4-hydroxytamoxifen sulfamates as estrone sulfatase inhibitors, but their apparent IC_{50} values are >30 μM. 4-Methylcoumarin-7-*O*-sulfamate has also been identified as a potent sulfatase inhibitor devoid of estrogenic activity. This agent was orally active in rats and after multiple doses (10 mg/kg/day for 7 days); liver estrone sulfatase activity was inhibited by 85% (Purohit *et al.*, 1996). Another less potent inhibitor is danazol, an agent used for treating endometriosis (Kauppila, 1993). However, its use is limited by androgenic side effects. The search for novel, selective and safe estrone sulfatase inhibitors is one of the goals of our program.

Estrone sulfatase is prevelant in mammalian liver, testis, adrenal, and ovary (Hobkirk, 1985). The intracellular distribution of the enzyme was studied in rat liver and testis, and the highest concentration was found in the microsomal fraction. Sulfatase has also been found to be amplified in NMU-induced rat tumors compared to normal mammary glands, as well as mammary cancer cell lines (Masamura *et al.*, 1996; Pasqualini *et al.*, 1996).

In vivo studies involving sulfatase inhibitors have employed measurement of estrone sulfatase activity in WBC and liver samples (Purohit *et al.*, 1996). Agents have also been shown to completely block the ability of estrone sulfate to stimulate uterine growth in ovariectomized rats. In addition, studies were performed with Ludwig rats. Following three injections with NMU (50 mg/kg), mam-

mary tumors developed after 4-5 months. The animals were then ovariectomized and approximately 70% of the hormone-dependent tumors regressed. Regrowth of the tumors was stimulated with estrone sulfate for 19 days, and estrone sulfamate was shown to reduce the regrowth of these estrone sulfate-stimulated NMU-induced tumors (Purohit *et al.*, 1995).

Before searching for new inhibitors, preliminary evaluations were carried out to standardize estrone sulfatase assay parameters. This constitutes an important prerequisite for the valid comparison of enzyme inhibitors, since the concentrations of reagents used (especially those of the enzyme and substrate) determine the catalytic efficiency of the enzyme, and ultimately its susceptibility to inhibition by agents with varied mechanisms of action. For the model system, we utilized partially purified female Sprague-Dawley rat liver microsomes as the source of estrone sulfatase. Tritiated estrone sulfate is used as the substrate, and the product, tritiated estrone, is separated from the substrate using a two-phase scintillation system in which the polar substrate remains in the aqueous layer and the nonpolar products partition into the organic layer (MacIndoe, 1988). Studies were performed to assess the effects of experimental variables such as reaction time, enzyme, substrate and DMSO concentrations on the formation of the final product, estrone. The sulfatase assay was subsequently established by utilizing subsaturation conditions with respect to all these parameters. Under these conditions, partially purified enzyme should demonstrate optimum sensitivity to inhibitors acting by diverse mechanisms of action.

Utilizing the standardized reaction conditions, more than 50 compounds were evaluated for inhibition of sulfatase activity. As summarized in Table I, hederagenin, ellagic acid, oleanolic acid, ursolic acid, and betulinic acid were the most potent sulfatase inhibitors tested, and dose-response curves were generated and IC_{50} values calculated. Most active compounds were triterpenoids such as ursolic acid, which was assessed further for the inhibition of estrone sulfatase in microsomes of MCF-7 cells, and rat mammary tumor homogenates (Santen *et al.*, 1986). With each of these preparations, ursolic acid was equipotent in inhibiting estrone sulfatase activity. The IC_{50} values are summarized in Table II. Santen *et al.* (1986) have shown that estrone sulfate causes an increase in colony formation of NMU tumor cells grown in soft agar under serum-free conditions. Using this same model, we have found that ursolic acid inhibits the induction of colony formation (Fig. 2), providing additional evidence that ursolic acid is indeed acting through inhibition of estrone sulfatase. However, other mecha-

Table I. Effect of potential chemopreventive agents on the activity of rat liver estrone sulfatase[1]

Agent[2]	IC_{50} (μg/ml)[3]
Betulinic acid	0.4
Carbenoxolone	2.2
Ellagic acid	4.7
Oleanolic acid	0.12
Ursolic acid	0.08

[1]Danazol was used as positive control : IC_{50} = 2.3 μg/ml.
[2]All agents were purchased from Sigma Chemical Co. (St. Louis, MO).
[3]The method of MacIndoe (1988) was used for identifying active compounds. Agents were initially tested at a concentration of 40 μg/ml and those exhibiting ≥ 50% inhibition were tested at different concentrations to obtain dose-response curves, and IC_{50} values were calculated as described previously (Jeong *et al.*, 1999).

Table II. Inhibitory effects of ursolic acid on various estrone sulfatase preparations.

Source	IC_{50} (μg/ml)[4]
Rat Liver Cytosol[1]	0.08
MCF-7 Cytosol[2]	0.12
DMBA Mammary Tumor Homogenates[3]	0.06
NMU Mammary Tumor Homogenates[3]	0.2

[1]Rat liver cytosolic preparations were obtained as described by MacIndoe (1988).
[2]The method of Nguyen *et al.* (1993) was used. Briefly, MCF-7 cells were routinely maintained in minimal essential medium with Earle's salts (MEME) supplemented with FBS (5%), streptomycin (100 μg/ml), penicillin (100 U/ml), insulin (10 μg/ml), l-glutamine (2 mM), sodium pyruvate (1 mM), and nonessential amino acids at 37°C in a 5% CO_2 incubator. One week before plating the cells, the medium was changed to phenol red-free formulation of MEME containing twice-stripped FBS. Preconfluent cells (three flasks of 75 cm^2) were washed twice with ice-cold Hank's Balanced Salt Solution (HBSS) and harvested by gentle scraping. After centrifugation at 900 g for 10 min, the pellets were incubated for 10 min with cold 1.5 mM $MgCl_2$, homogenized and diluted (1:1, v/v) with 0.04 M Tris-HCl buffer, pH 6.5. The homogenate was centrifuged at 200,000 g for 30 min; the supernatant was used as the enzyme source after protein determination (Kellis and Vickery, 1987).
[3]The method of Santen *et al.* (1986) was used. Briefly, DMBA- and NMU-induced rat tumors were obtained 30 min after surgical excision, frozen in liquid nitrogen, pulverized, and stored at 70°C until use. For the DMBA-induced tumors, 50 day-old virgin female Sprague-Dawley rats were given a single dose of DMBA (15 mg) in sesame oil following an overnight fast. NMU-tumors were induced by administering single i.v. injection of NMU in 6.5% ethanol, 93.5% neobee oil to 49 day-old female Sprague-Dawley rats. The tissues were homogenized in 4 vol of potassium phosphate buffer (20 mM K_3PO_4 containing 1 mM EDTA, pH 7.5). Homogenates were used as the enzyme source after protein determination (Kellis and Vickery, 1987).
[4]IC_{50} values were calculated as described in Table I.

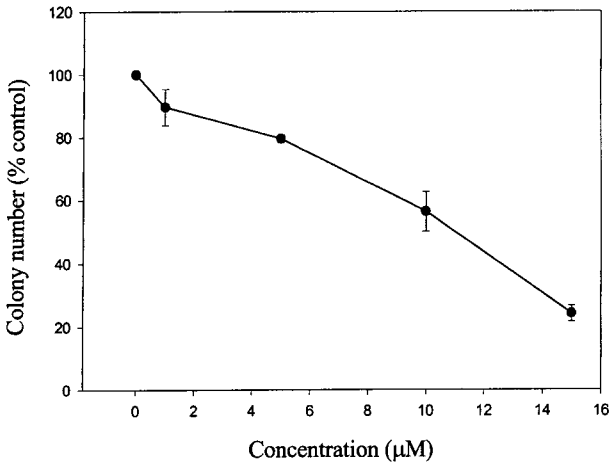

Fig. 2. Effect of ursolic acid on NMU-induced tumor colony formation in soft-agar. Soft-agar culture provides an efficient means of growing excised NMU tumors, as established by Manni and Wright (1983), when 16 of 16 separate neoplasms grew in dishes. Addition of estrone sulfate (1 μM) increased the number of colonies to 58 ± 5.6 colonies/dish from 13 ± 2.3 colonies found in the control group (without estrone sulfate). Incubation of the cell population with ursolic acid did not affect the colony size (data not shown). Ursolic acid dose-depen-dently decreased the number of colonies with an IC_{50} value of ~10 μM.

nisms of inhibition of mammary tumors by ursolic acid seem to exist. For example, ursolic acid exhibits both cytostatic and cytotoxic activity by arresting MCF-7 cells in the G_1 phase of the cell cycle at a concentration of 20 μM (Es-Saady *et al.*, 1996).

Triterpenoids exist widely in nature and are used for medicinal purposes in many Asian countries. Ursolic acid possesses anticancer and anti-inflammatory effects, and also inhibits TPA-induced inflammation and tumor promotion in mouse skin (Huang *et al.*, 1994; Manez *et al.*, 1997). In a recent study, ursolic acid was shown to be a specific inhibitor of cyclooxygenase-2 (COX-2) expression and transcriptional activation in human mammary epithelial cells (Subbaramaiah *et al.*, 2000). Further, Paik *et al.* (1998) suggest that ursolic acid exhibits differentiation-inducing effects on rat mammary epithelial cells in primary or in matrigel culture. In summary, our studies provide evidence of estrone sulfatase involvement in tumor cells and the inhibition of this enzyme by ursolic acid suggests a mechanistic basis for the mammary cancer chemopreventive potential of this agent.

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