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Phytochemicals for breast cancer prevention by targeting aromatase

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1. ABSTRACT

Aromatase is a cytochrome P450 enzyme (CYP19) and is the rate limiting enzyme in the conversion of androgens to estrogens. Suppression of *in situ* estrogen production through aromatase inhibition is the current treatment strategy for hormone-responsive breast cancers. Drugs that inhibit aromatase have been developed and are currently utilized as adjuvant therapy for breast cancer in post-menopausal women with hormone dependent breast cancer. Natural compounds have been studied extensively for important biologic effects such as antioxidant, anti-tumor and anti-viral effects. A significant number of studies have also investigated the aromatase inhibitory properties of a variety of plant extracts and phytochemicals. The identification of natural compounds that inhibit aromatase could be useful both from a chemopreventive standpoint and in the development of new aromatase inhibitory drugs. This review will discuss whole food extracts and the common classes of phytochemicals which have been investigated for potential aromatase inhibitory activity. We will review reported aromatase inhibition, kinetic data and possible structural variations that may inhibit or enhance the interaction of phytochemicals with the aromatase enzyme.

2. INTRODUCTION

Chemoprevention involves the use of natural or synthetic substances to prevent a disease such as cancer. The objective of chemoprevention, therefore, is to delay or prevent the instigation of cancer, its progression or its reoccurrence after treatment is completed. Breast cancer is the second leading cause of death in women and is most common in post-menopausal women. Approximately 80% of breast cancers are estrogen receptor (ER) positive and respond to the growth promoting effects of estrogen (1). In post-menopausal women, estrogen is no longer produced by the ovaries but in peripheral tissues such as adipose and breast tissue. Therefore, despite decreased circulating estrogen (plasma), local estrogen levels in the breast can persist at similar levels to that of pre-menopausal woman (2, 3) and estrogen levels in breast tumors can be up to 40 times higher than circulating estrogen (4).

Aromatase is a cytochrome P450 enzyme (CYP19) and is the rate limiting enzyme in the conversion of androgens to estrogens. Aromatase is expressed in the bone, ovary, brain, skin, placenta and adipose tissue and is expressed at higher levels in breast tumors and surrounding cells when compared to non-cancerous breast cells (5, 6).

Approximately 70% of breast cancer cells produce estrogen through the expression of aromatase (7, 8). Therefore, suppression of *in situ* estrogen production through aromatase inhibition is a viable treatment strategy for hormone-responsive breast cancers. Drugs that inhibit aromatase, aromatase inhibitors (AIs) have been developed and are currently utilized as adjuvant therapy for breast cancer in post-menopausal women with hormone dependent breast cancer. AIs are not used in premenopausal patients due to the fact that the production of estrogen in the ovaries is under feedback regulation through the hypothalamus, and therefore aromatase inhibition in this case is ineffective. Drugs currently in use include the non-steroidal compounds Anastrozole and Letrozole and the steroidal compound Exemestane.

The increased efficacy of AI over tamoxifen therapy has been demonstrated by clinical trials, whereby a significant increase in disease-free survival has been shown using three third-generation AIs (9-12). In addition, these major clinical trials, including ATAC, BIG 1-98, MA 17 and IES, demonstrated a significant decrease in the cases of contralateral breast cancer in the AI treated versus tamoxifen arm. It is important to point out that AIs are thought to be of value in treating postmenopausal patients, where peripheral aromatase is not under gonadotropin regulation (13). In premenopausal women, luteinizing hormone (LH) and follicle stimulating hormone (FSH) stimulate the synthesis of aromatase in the ovaries and may counteract the effects of AIs. Although these drugs are proving to be effective in the treatment of post-menopausal breast cancer patients, the course of treatment is a maximum of five years and women at risk for breast cancer are not prescribed drugs as a preventive measure. Therefore, an alternate approach to chemoprevention following treatment or in women at risk of developing breast cancer could be one that focuses on the intake of a diet rich in anti-aromatase phytochemicals.

2.1. An Overview of the phytochemicals

Phytochemicals are plant-derived chemical compounds, and the phenolic compounds are the largest class of phytochemicals that have been investigated for potential anti-aromatase activity. In addition to the phenolics, some alkaloid compounds have also been identified that exhibit this ability as well. It is well known that phenolic compounds are potent dietary antioxidants (14-16); however research has shown that in disease prevention, phenolic compounds potentially possess activity beyond that of anti-oxidation. For example, a variety of these compounds have been shown to alter the activity of cell receptors and signaling pathways (17-19), cell cycle and cell death pathways (20, 21) and inhibit enzymes that are linked to disease processes such as cyclooxygenase-2 (COX-2) (22) and lipoxygenase (LOX) (23).

2.1.1. Phenolic compounds

Phenolic compounds contain a basic aromatic ring structure and based on the number of phenolic rings and other elements such as methyl groups, oxygen atoms and sugars in the structure, they can be classified into different groups. The main groups are the flavonoids, phenolic acids, phenolic alcohols, stilbenes and lignans (24, 25). The flavonoids are the largest group of phenolic compounds, which can be broken down into six sub-groups: flavanones, flavones, isoflavones, flavonols, flavanols, and the anthocyanins and proanthocyanidins (26).

2.1.1.1. Flavonoids

Flavonols are the most ubiquitous phenolic compounds in plant species. The most common sources of flavonols include: broccoli, blueberries, kale, tea and wine. Flavonols contain a double bond between carbons 2 and 3 and a hydroxyl group at carbon 3. The most abundant flavonols found in plants are quercetin and kaempferol (24, 26). The basic chemical structures of the flavonoids, androstenedione and the AI aminoglutethimide can be seen in Figure 1.

Flavones, which also contain a double bond between carbon 2 and 3, are less widespread than flavonols. The largest dietary sources of flavones include celery and parsley, which are eaten in small amounts if at all in the average diet. Prenylflavonoids, a class of flavones that contain a hydrophobic group attached to the basic flavonoid structure, can be found in hops and therefore beer (27). The list of flavones tested for anti-aromatase activity includes: apigenin, biochanin-A, β-napthoflavone, chrysin, flavone, luteolin, rotenone and rutin. The prenylflavones are 8-prenylnaringenin, isoxanthohumol and xanthohumol.

Flavanones differ from flavones in that there is a single bond between C-2 and C-3, they also contain an oxygen atom at carbon 4 and are generally glycosylated at carbon 7 by a disaccharide. Common dietary sources of flavanones include tomatoes, mint and citrus fruits. The most abundant dietary flavanones are naringenin, hesperitin and eriodictyol (24). Potential aromatase inhibiting flavanones are flavanone, hesperetin and naringenin.

Isoflavones are an interesting class of flavonoids in that they are structurally very similar to estrogen and have been shown to bind to the estrogen receptor (ER) (28, 29) and elicit weak estrogenic and antiestrogenic effects (30-32). Due to their similarity to estrogen and their estrogenic activity, isoflavones are classified as phytoestrogens. The three most abundant isoflavones are genistein, daidzein and glycitein, found mainly in leguminous plants such as soy (24). The isoflavones that have been investigated for aromatase inhibition include: daidzein, equol, genistein and 0-desmethylangolensin.

Anthocyanins are compounds that are mainly concentrated in the skins of fruits and vegetables and are responsible for their red, purple and blue colors. They exist principally as glycosides called anthocyanidins and are found in a wide variety of fruits such as grapes, berries, apples as well as in wine and tea (24). Anthocyanins have not, to date, been investigated for their effects on aromatase.

Flavanols are different from other flavonoids in that they are not glycosylated. The main flavanols include catechin and epicatechin (found primarily in fruit) and

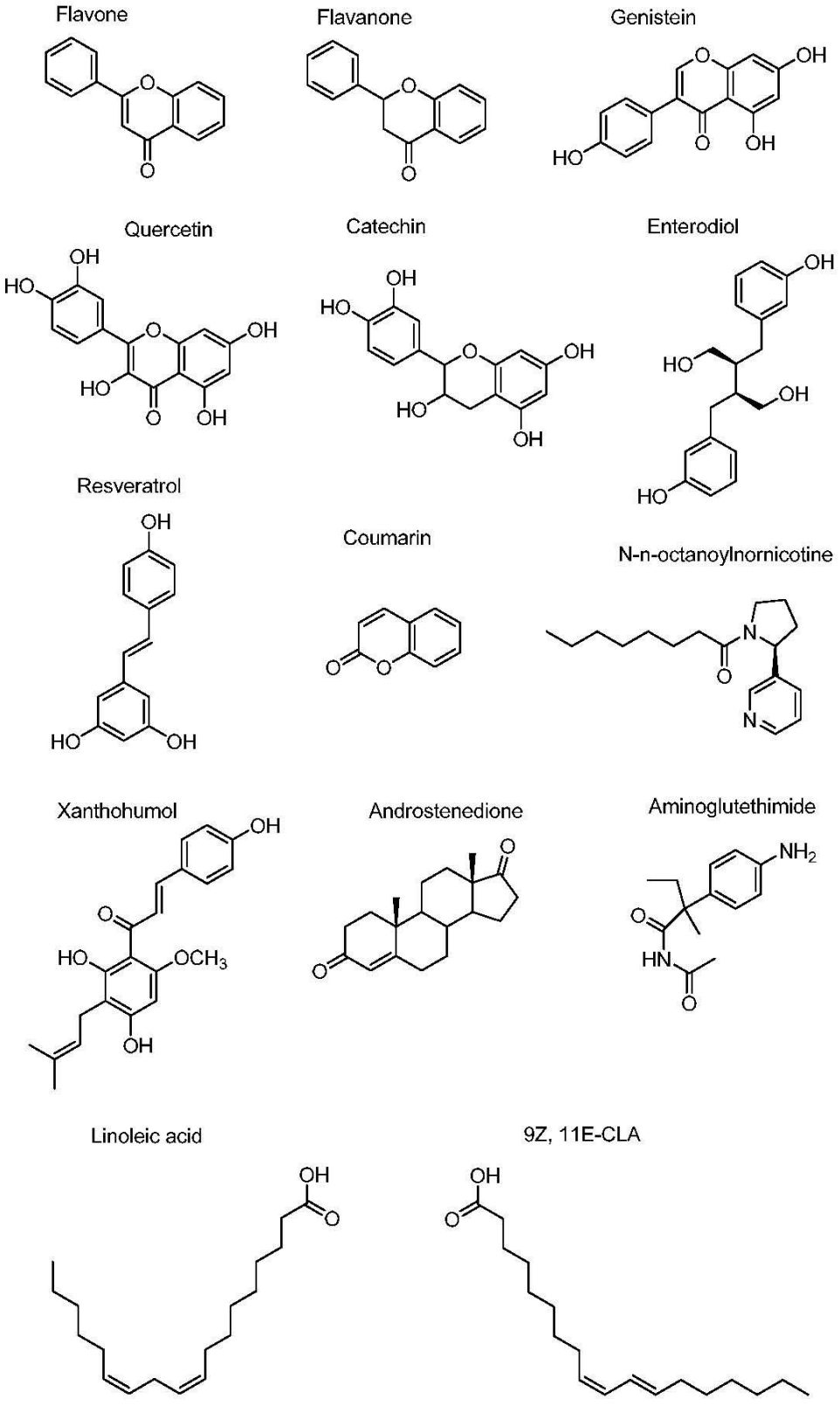

Figure 1. Representative structures of each class of phytochemical, androstenedione (substrate of aromatase), aminoglutethimide (aromatase inhibitor), linoleic acid and CLA.

epigallocatechin and egpigallocatechin gallate (found primarily in tea) (24, 26). The flavanols butein, catechin and its various forms and theaflavins have been tested in aromatase inhibition assays.

2.1.2. Non-flavonoid compounds.

The non-flavonoid phenolic compounds of interest as bioactive phytochemicals include: caffeic acid phenethyl ester (CAPE) which is isolated from bee propolis, curcumin (Curcuma longa) found in turmeric, stilbenes (such as resveratrol) and lignans and quinones (such as emodin and hypericin) (26). Stilbenes are produced in plants in response to stress and are widely found in plants including grapes, berries, peanuts, red wine and grape juice. Stilbenes are similar to flavonoids in their biosynthetic pathway and may be referred to as stilbenoids in the literature for this reason (26). Resveratrol is a stilbene that has been found not only to exhibit anti-cancer activity (33) but to decrease inflammatory pathways and inhibit platelet aggregation (26). The structure of resveratrol can be seen in Figure 1.

Lignans are diphenolic compounds produced from plant precursors (mainly secoisolariciresinol and matairesinol) by the action of intestinal bacteria and are one of the major classes of phytoestrogens. Enterolactone and enterodiol are the main lignans produced in the mammalian system (34) and an example of the structure of enterodiol can be observed in Figure 1. Coumarins are non-flavonoid phenolic compounds that are derived from cinnamic acid and found in a wide variety of plants. A variety of coumarins and their derivatives have been examined for their anti-aromatase activity, however very few have been found to be active. The basic structure of coumarin can be seen in Figure 1.

One class of non-phenolic compounds identified as anti-aromatase phytochemicals are alkaloids. Over 2200 compounds have been identified in tobacco and tobacco smoke, although nicotine, nornicotine and anabasine are the most common (35). Two alkaloid compounds isolated from tobacco leaves and smoke (N-n-octanoylnornicotine and N-(4-hydroxyundecanoyl) anabasine have been shown to demonstrate anti-aromatase activity (36). In addition, through studies of white button mushroom extract, the unsaturated fatty acids linoleic and conjugated linoleic acid (CLA) were identified as AIs as well (37). The fatty acid compounds will be discussed with the white button mushroom studies and their structures can be seen in Figure 1.

3. ANTI-AROMATASE ACTIVITY OF WHOLE PLANTS

3.1. Grapes and grape seed extract

Although most studies investigating natural AIs focus on single isolated compounds, the investigation of the aromatase inhibitory activity of whole foods has been done to a limited degree. In one of the first studies, a panel of fruit juices was tested in a human placental microsome assay. Results from this study showed that of the juices tested (orange, peach, grapefruit, plum, apple, strawberry, red seedless grape), the red seedless grape juice inhibited aromatase activity to the highest degree. The authors then focused on different types of grapes and their studies showed that black grapes, green seedless grapes and an extract of grape seed extract also significantly inhibit aromatase activity.

Kinetic analysis of the green seedless grape juice showed that the inhibition was competitive with respect to the substrate, androstenedione. Results of cell culture studies done with the MCF-7aro aromatase over-expressing cell line showed that although treatment with grape juice did not inhibit estradiol or testosterone-induced cell proliferation, there was a synergistic inhibitory effect when combined with Tamoxifen. This suggests that dietary or supplemental intake of grape phytochemicals could potentially improve response to Tamoxifen treatment in breast cancer patients, although this hypothesis would have to be confirmed in an *in vivo* system. In addition, in-cell assays are useful in that cellular uptake and metabolism of the compounds occurs and can therefore give a truer picture of how the compounds would act in a living system. Finally, the intake of 500 μL of grape juice per day in an MCF-7aro xenograft mouse model was shown to decrease tumor size 70% compared to untreated controls (38). Although no inhibition of breast cancer cell proliferation was observed *in vitro*, the observed decrease in tumor growth in mice suggests that grape juice phytochemicals possess true anti-cancer activity *in vivo*.

Because wine is a product of grapes, further study by this laboratory investigated the effect of wine on aromatase activity *in vitro* and *in vivo*. In a comparison of different types of wines, it was found that red wines are more potent inhibitors of aromatase activity in the placental microsomal assay compared to white wines. Red wine phytochemicals were separated via liquid chromatography and the resulting fractions were tested for anti-aromatase activity. It was determined that the 20% acetonitrile (ACN) fraction was the most potent aromatase inhibitor and that this fraction inhibited aromatase in a competitive manner. Further experiments in transgenic aromatase over-expressing mice demonstrated that daily gavage of the ACN wine fraction inhibited aromatase-induced mammary hyperplasia in this mouse model (39), again demonstrating the in-*vivo* activity of grape phytochemicals.

In a separate study, whole red wine was fractionated and the isolated fractions tested in a placental microsomal assay. The fraction with the highest anti-aromatase activity was isolated and identified as a procyanidin B dimer. Using mass spectral analysis, the authors determined that the procyanidin B dimer isolated from red wine contained a 4-6 linkage, however they were not able to determine the exact isomer. The procyanidin dimer was determined to be a competitive inhibitor of aromatase with a Ki value similar to that of the positive control, the aromatase inhibitor aminoglutethimide (AG). Utilizing aromatase mutagenesis studies, it was found that Asp-309, Thr-310, Ser-478 and His-480, all of which are contained within the active site of aromatase, are important to the inhibition of aromatase by procyanidin B dimer. Finally, studies utilizing the MCF-7aro xenograft nude

mouse model illustrated that the 60 – 80% methanol fraction of red wine, which contains mainly procyanidin B dimers, significantly reduced tumor size when compared to control mice (40). These studies clearly show the efficacy of procyanidin B dimers isolated from red wine in the inhibition of aromatase *in vitro* and *in vivo*.

Grape seeds also contain potent phytochemicals of which, procyanidin B dimers are a major component. A commercially available grape seed extract (GSE) was found to contain approximately 78% proanthocyanidins (41). A study into the anti-aromatase activity of GSE showed that GSE inhibited aromatase in a dose-dependent manner in a placental microsome assay. In addition, GSE treatment in MCF-7 and SKBR-3 breast cancer cell lines decreased exon I.3 and exon II-containing aromatase mRNA expression. These are the major exon I's isolated from breast cancer tissues, suggesting that promoters I.3 and PII are the primary promoters that induce aromatase expression in breast cancers. In contrast, aromatase expression in normal breast tissue is driven primarily by promoter I.4 (41). In the MCF-7aro breast cancer xenograft model, GSE treatment significantly decreased androgen-induced tumor growth (41). This study illustrates the ability of the phytochemicals in GSE to inhibit aromatase *in vitro* and *in vivo*. Overall, the intake of grape juice, wine or supplementation with commercially available grape seed extract may therefore aid in the prevention of estrogen-dependent breast cancers.

3.2. White button mushroom

A more unassuming food that may be useful in the fight against breast cancer is the white button mushroom (*Agaricus bisporus*). In a study which screened heat stable extracts of common vegetables against aromatase activity in a placental microsomal assay, the white button mushroom showed the highest activity. Further screening of different types of mushroom species produced results indicating that with the exception of the stuffing mushroom, the white button mushroom had the highest activity. Kinetic analysis demonstrated that white button mushroom extract exhibits a mixed type of inhibition, indicating either more than one type of inhibitor in the mushroom extract, or more than one mechanism of inhibition. In-cell aromatase assays utilizing the MCF-7aro aromatase over-expressing cell line showed that mushroom extract is capable of inhibiting both testosterone-induced breast cancer cell proliferation and aromatase activity (42).

Further experiments involved fractionating the mushroom extract into an aqueous and an ethyl-acetate (EA) fraction. Both total fractions significantly inhibited aromatase activity in the human placental microsome assay. The fractions were further purified and it was found that the EA-2 fraction was the most potent. Therefore, EA-2 was further fractionated and although all of the EA-2 sub-fractions significantly inhibited aromatase activity, the total EA-2 fraction was more potent. Analysis of the composition of the EA-2 fraction showed that the major active components were linoleic and linolenic acids. To quantify the relative amounts of fatty acids in the EA fraction, gas chromatography was utilized. Conjugated linoleic acid (CLA) (c9t11) was identified as 45.7% of the active fraction with 21% identified as linoleic acid. Linoleic acid and CLA were both shown to be non-competitive inhibitors of aromatase in kinetic analyses. Studies utilizing mutant aromatase constructs showed a decreased interaction of linoleic acid with aromatase when His480 and Ser478 were mutated. Alternatively, Glu302 mutation facilitated the interaction, illustrating the importance of these amino acids to the interaction of linoleic acid with the aromatase enzyme.

Cell analysis utilizing the MCF-7aro cell line showed that both mushroom extract and CLA suppressed androgen-induced cell proliferation. Mushroom extract also significantly decreased androgen-dependent MCF7-aro xenograft tumor growth in nude mice. Histologic analysis of the tumors showed decreased cell proliferation in tumors from mushroom-fed mice compared to untreated controls (37). These studies suggest that daily intake of the common white button mushroom may have a significant chemopreventive effect with regard to breast carcinogenesis. White button mushrooms are relatively inexpensive and readily available in markets across the United States, and therefore are a feasible addition to any dietary plan.

3.3. Pomegranate

Pomegranate (*Punica granatum* L.) is a fruit that is rich in polyphenols and is commonly consumed either as fresh fruit, jam or juice. Pomegranate juice is enriched in tannins and has been shown to demonstrate antioxidant, antisclerotic and anti-cancer properties (43-45). Studies into the anti-aromatase activity of pomegranate juice, fermented juice and fermented juice and pericarp extracts were recently undertaken utilizing a placental microsome assay. This study found that the polyphenols in both fermented juice and pericarp significantly inhibited aromatase activity when compared to the positive control, aminoglutethimide (51% and 24% of the activity of positive control, respectively). In addition to anti-aromatase activity, fermented juice also inhibited MCF-7 and MDA-MB-231 breast cancer cell proliferation, to a greater degree than the fresh juice. Pomegranate seed oil was also shown to inhibit cell proliferation and the invasive activity of MCF-7 cells. In addition, fermented pomegranate juice inhibited the formation of DMBA-induced breast lesions in a murine mammary organ culture (46). These studies not only identify polyphenols from pomegranates as potent anti-aromatase phytochemicals, but demonstrate their ability to inhibit breast carcinogenesis *in vivo*.

3.4. Herbal extracts

Extracts of the herb *Scutellaria barata* D. Don (SB) and the invasive shrub *Euonymus alatus* Sieb (EA) were also shown to possess anti-aromatase activity. SB is an herb native to China and has been utilized in traditional Chinese Medicine as an anti-inflammatory and anti-tumor treatment. SB contains high levels of both alkaloids and flavone compounds (47). EA is a traditional Korean medicine that has be used as an anti-tumor agent in both Korea and China (48). EA is known to contain a variety of

flavonoids, cardenolides, caffeic acid (48) and recently quercetin and rutin were extracted from this plant (49). Extracts of both of these herbs were tested in aromatase expressing myometrial and leiomyomal cells and also in human placental tissue. Both of the extracts were found to exhibit anti-aromatase activity, with EA showing 10-30 times more activity in the "in-cell" assays than SB (50). Therefore, extracts from either of these plants could potentially be utilized for chemoprevention through inhibition of the aromatase enzyme. Further study *in vivo* is needed to determine the relative usefulness of these botanical extracts.

4. FLAVONOID COMPOUNDS

4.1. Flavones

The largest group of flavonoids reported to exhibit anti-aromatase activity is the flavones. Early studies were undertaken by Kellis and Vickery in their investigations into the anti-aromatase activity of natural and synthetic flavones. Utilizing a placental microsomal assay, the authors tested seven flavones, two synthetic (7,8-benzoflavone and 5,6-benzoflavone) and five natural (chrysin, apigenin, flavone, flavanone and quercetin) with AG as a positive control. The most effective compound proved to be 7,8-benzoflavone (α-naphthoflavone) (IC_{50} 0.07 nM), which was shown to be ten times more potent as AG. Binding studies revealed that α-naphthoflavone bound aromatase half as tightly as the substrate androstenedione and had a slightly higher affinity than testosterone. Kinetic analysis proved this compound to be a competitive inhibitor of aromatase. The remainder of the compounds tested were effective in this order: chrysin (IC_{50} 0.5 nM), apigenin (IC_{50} 1.2 nM), flavone (IC_{50} 8 nM), flavanone (IC_{50} 8 nM) and quercetin (IC_{50} 12 nM) with 5,6-benzoflavone showing no activity in the assay. Chrysin, as the second most potent inhibitor, was also tested in the kinetic assay and found to be a competitive inhibitor, leading the authors to conclude that flavones compete with the substrate (androstenedione or testosterone) for binding to the active site of aromatase (51).

Further studies utilizing spectral analysis showed that 7,8-benzoflavone caused a change in the spectra, indicating a conversion of the heme iron in aromatase from a high to a low spin rate (i.e.: displacement of the substrate and formation of an aromatase-7,8-benzoflavone complex). When chrysin was substituted for 7,8-benzoflavone, the same spectral shift was observed. Based on their results, the authors speculated that flavones could either compete directly with the substrate for binding to aromatase or there may be a separate site on the enzyme that the flavones bind, causing a conformational change in the enzyme, thus decreasing the affinity of the substrate for the enzyme (51). These studies were of the first to show that natural compounds could potentially be utilized as aromatase inhibitors.

Ibrahim *et al*, in similar studies utilizing human placental microsomes, identified five flavones that inhibited aromatase activity with an IC_{50} of 10 μM or below: 7-hydroxyflavone (IC_{50} 0.5 μM), 7,4'-dihydroxyflavone (IC_{50} 2 μM), flavone (IC_{50} 10 μM), flavanone (IC_{50} 8 μM) and 4'-hydroxyflavanone (IC_{50} 10 μM). The authors concluded that flavones and flavanones exhibit greater anti-aromatase activity than the isoflavones and isoflavanones which exhibited much lower potency showing an EC_{50} range of 80 – 200 μM (52).

Investigations into the structure of the active flavone compounds led to the conclusion that 4'-hydroxylation of the B ring had a minor effect on aromatase inhibition with the exception of flavone, where hydroxylation at this point decreased activity significantly. In addition, hydroxylation at the 3 position of the C ring significantly decreased inhibitory activity, and reduction of the carbonyl group in flavone to a 4-hydroxyl group completely abrogated activity (52). These results are in agreement with those of the earlier studies done by Kellis and Vickery (51). Kinetic analysis showed 7-hydroxyflavone to be a competitive inhibitor of aromatase, and spectroscopic analysis results were in agreement with the previous results by Kellis and Vickery (51) in that they showed 7-hydroxyflavone caused displacement of the substrate (androstenedione) (52).

Similar studies utilizing a preadipocyte cell culture system also identified α-naphthoflavone as a highly potent inhibitor of aromatase (IC_{50} of 0.5 μM). In addition, chrysin, flavone and Biochanin A were also identified as active; however the activity of flavone and biochanin A was marginal (IC_{50} of 68 and 113 μM, respectively). In contrast to alpha-napthoflavone, β-naphthoflavone did not exhibit any inhibitory activity (53). Jeong *et al* also found the flavones apigenin, chrysin and hesperetin to have anti-aromatase activity in the human placental microsome assay (54). Le Bail *et al* had similar results, showing that flavone, 7-hydroxyflavone, 7-methoxyflavone, 7,8-dihydroxyflavone, chrysin and apigenin all inhibited aromatase (55).

Four flavones (chrysin, 7,8-dihydroxyflavone, baicalein and galangin) were tested for anti-aromatase activity by Kao *et al* (56) and it was found that bailcalein did not inhibit aromatase as concentrations of 50 μM and below. The Ki values of the compounds that did show activity were as follows: chrysin (2.6 μM), 7.8-dihydroxyflavone (10 μM) and galangin (95 μM). A previous study by this same group also showed chrysin to be a more potent inhibitor than 7,8-dihydroxyflavone with IC_{50} values of 11 and 55 μM, respectively (57). Based on mutagenesis studies, the authors predict that the active flavones bind to the aromatase enzyme where the A and C rings of the flavone mimic the C and D rings of substrate. Mutagenesis studies also suggested that the hydroxyl group at C-8 of 7,8-dihydroxyflavone decreases its binding affinity (compared to chrysin which contains a hydroxyl group at C-5). Galangin, which has a hydroxyl group at C-3 did not inhibit aromatase in these studies, indicating that a hydroxyl group at this location significantly inhibits binding to the enzyme (56). These results give important insight into the relationship of inhibitor structure to the binding and inhibition of aromatase.

A recent study by Sanderson *et al* utilized adrenocortical carcinoma cells (H295R) to test aromatase inhibition by several natural and synthetic flavones. This study found the flavone rotenone to be the most potent aromatase inhibitor (IC_{50} of 0.3 μM) after 24 hours of exposure. In agreement with the results of other investigators, 7-hydroxyflavone, chrysin and apigenin also exhibited anti-aromatase activity. This study was the first to find anti-aromatase activity of rotenone, a natural pesticide in tropical plants that is known to inhibit mitochondrial function. The authors speculated on the anti-aromatase mechanism of the compounds tested and suggest that the oxo-group on the 4 position of the C ring is essential to the inhibitory activity of flavones. In addition, flavones hydroxylated on the A ring tended to be more potent than those hydroxylated in the B ring (58).

Recent studies utilized a co-culture system where MCF-7 breast cancer cells were cultured with fibroblasts to mimic the interaction between tumor cells and the surrounding tissue. The authors utilized this method to do in cell aromatase assays and found that the flavones apigenin, chrysin and biochanin A all inhibited aromatase activity in this system, however the IC_{50} for biochanin A was 25 μM, showing it to be a moderate inhibitor compared to apigenin (IC_{50} 4.1 μM) and chrysin (1.5 μM) (59).

Biochanin A was also shown to have significant anti-aromatase activity in MCF-7aro cells. A recent study showed that not only did this compound inhibit aromatase mRNA expression but it also decreased aromatase promoter I.3 and II activity in reporter gene assays. An interesting observation from this study was the finding that although genistein (a major metabolite of biochanin A) did not inhibit aromatase, it did decrease promoter activation. The authors speculate that the suppressive effect of biochanin A on aromatase may be due, in part, to its metabolism to genistein (60).

Hop prenylflavonoids and different types of beer (which contain hop prenylflavonoids) have also been investigated for their anti-aromatase activity in choriocarcinoma-derived JAR cells. The most potent of the compounds tested was 8-prenylnaringenin (8-PN) with an IC_{50} of 0.065 μM, where xanthohumol (XN) had an IC_{50} of 20.3 μM and isoxanthohumol (IXN) was a weak inhibitor at 139.7 μM. Interestingly, none of the compounds had an effect on the expression of aromatase mRNA as determined by RT-PCR. When the compounds were tested on recombinant aromatase at the IC50s determined with the in-cell assay, only IXN inhibited recombinant aromatase. The other compounds had to be increased to a concentration ten times that of the IC_{50} in JAR cells, suggesting that cellular metabolism converts the compounds into other, more active components (27).

The compounds in this study exhibiting the highest anti-aromatase effect contain a carbonyl group on C4 of the C ring. In addition, the most active compound, 8-PN, also contains a 5-hydroxyl group which is similar to the 4-hydroxyl group of androstenedione (27). However, because the individual compounds exhibited anti-aromatase activity in the in-cell assay only, it is possible that they are metabolized into other compounds and that these structural details may be altered. Whole beer products were also tested in JAR cells. The lager beer (LB), stout beer (SB) and xanthohumol-rich stout beer (XSB) all inhibited aromatase activity without affecting cell viability. XSB exhibited the strongest inhibition (73.6% inhibition), with SB and then LB having a more modest effect (66.1% and 24.2%, respectively) (27).

The same group later repeated these experiments using the SKBR-3 breast cancer cell line and in agreement with their earlier study in JAR cells, found 8-PN to be the most active compound (IC_{50} of 0.08 μM) where XN and IXN had IC_{50}'s of 3.2 μM and 25.4 μM, respectively. All three compounds significantly inhibited cell proliferation and induced apoptosis in SKBR-3 cells, possibly due to the inhibition of aromatase activity in these cells (61).

Chalcones are flavonoid precursor compounds found in a variety of plants. One study tested a panel of chalcones for anti-aromatase activity in the MCF-7aro cell line. Of the five chalcones tested, 2-hydroxychalcone, 2'-hydroxychalcone, 4-hydroxychalcone, 4,2',4'-trihydroxychalcone (isoliquiritigenin found in licorice) and 3,4,2',4'-tetrahyrdoxychalcone (butein), butein exhibited the strongest anti-aromatase activity with an IC_{50} value of 3.75 μM. Kinetic analysis of the interaction between butein and aromatase revealed a mixed-type of inhibition and proliferation assays showed decreased testosterone-induced cell proliferation with butein treatment compared to untreated controls (62). This illustrates that the flavonoid precursor, butein, can also act as an AI and possible chemopreventive agent.

Overall, these studies show that flavones may be the most potent natural inhibitors of aromatase activity both in cell free and in-cell assays. The IC_{50} values of the flavones from reported studies are listed in Table 1. From the above reports it appears that the most active compounds are α-naphthoflavone (a synthetic flavone) with 7-hydroxyflavone, apigenin, biochanin A, chrysin and flavone also showing significant activity. Other compounds such as 7,4'-dihydroxyflavone and 7,8-dihydroxyflavone also showed activity whereas others like 5,6-benzoflavone and 7-methoxyflavone did not.

Neves *et al* found that of the panel of polyphenolic compounds they tested, the only ones with activity equal to or greater than the control (Aminoglutethimide) were flavone or flavanone derivatives (64). Clearly, the basic structure and the number and position of hydroxyl and accessory groups in the structure of flavones have significant effects on its activity. A number of excellent recent studies have been published that go into the fine details of flavone structure as it relates to the anti-aromatase activity of these compounds (63, 64). An in-depth discussion of these findings in total would be beyond the scope of this review; however, a few general conclusions can be made based on the current knowledge. First, flavonoids may bind to the active site of the

Table 1. Reported inhibition of aromatase by flavones

Compound	Assay	IC_{50} (μM)	Reference
Apigenin	Placental microsome	1.2	(51)
Apigenin	Placental microsome	2.9	(55)
Apigenin	Adrenocortical carcinoma cell	20	(58)
Apigenin	Fibroblast/MCF-7 co-culture	4.1	(59)
Apigenin	Placental microsome	4.2	(63)
Apigenin	Placental microsome	3.33[1]	(54)
Baicalein	CHO cell expression	NE	(56)
Biochanin A	Preadipocyte cell	113	(53)
Biochanin A	Placental microsome	49	(55)
Biochanin A	Fibroblast/MCF-7 co-culture	25	(59)
Chrysin	CHO cell expression	2.6[2]	(56)
Chrysin	Preadipocyte cell	4.6	(53)
Chrysin	Placental microsome	4.33[1]	(54)
Chrysin	Placental microsome	0.5	(51)
Chrysin	Placental microsome	0.7	(55)
Chrysin	Adrenocortical carcinoma cell	7	(58)
Chrysin	Fibroblast/MCF-7 co-culture	1.5	(59)
Chrysin	Placental microsome	1.1	(63)
Flavone	Preadipocyte cell	68	(53)
Flavone	Placental microsome	10	(52)
Flavone	Placental microsome	8	(51)
Flavone	Placental microsome	48	(55)
Flavone	Adrenocortical carcinoma cell	NE	(58)
Flavone	Placental microsome	375	(66)
Galangin	CHO cell expression	95	(56)
Luteolin	Preadipocyte cell	25	(34)
Rotenone	Adrenocortical carcinoma cell	0.3	(58)
Rutin	Preadipocyte cell	NE	(53)
3',4',7-trihydroxyflavone	Placental microsome	NE	(55)
7,4'-dihydroxyflavone	Placental microsome	2	(52)
7,8-dihydroxyflavone	Placental microsome	8	(55)
7,8-dihydroxyflavone	CHO cell expression	10[1]	(56)
7-hydroxyflavone	Placental microsome	0.5	(52)
7-hydroxyflavone	Placental microsome	0.2	(55)
7-hydroxyflavone	Adrenocortical carcinoma cell	4	(58)
7-methoxyflavone	Placental microsome	3.2	(55)
7-methoxyflavone	Adrenocortical carcinoma cell	NE	(58)
α-napthoflavone (7,8-benzoflavone)	CHO cell expression	2.2	(56)
α-napthoflavone (7,8-benzoflavone)	Preadipocyte cell	0.5	(53)
α-napthoflavone (7,8-benzoflavone)	Placental microsome	0.07	(51)
β-napthoflavone (5,6-benzoflavone)	Preadipocyte cell	NE	(53)
β-napthoflavone (5,6-benzoflavone)	Placental microsome	NE	(51)
Catechin	Preadipocyte cell	NE	(53)
Catechin	Adrenocortical carcinoma cell	NE	(58)
Catechin gallate	Placental microsome	55	(65)
Epicatechin	Adrenocortical carcinoma cell	NE	(58)
Epigallocatechin	Placental microsome	100	(65)
Epigallocatechin gallate	Placental microsome	60	(65)
Epigallocatechin gallate	Placental microsome	13.79	(21)
Gallocatechin gallate	Placental microsome	15	(65)
Polyphenone-60	Placental microsome	28l	(65)
Theaflavin	Placental microsome	4.17	(21)
Theaflavin 3,3'-digallate	Placental microsome	3.45	(21)
Theaflavin 3-gallate	Placental microsome	3.23	(21)

NE=no effect, [1]molarity calculated from IC_{50} given in research paper in μg/ml. [2]Ki value.

aromatase enzyme such that the A and C rings mimic the D and C rings of the natural substrates (63). Hydroxylation of C-7 on Ring A and/or hydroxylation of both C-7 and C-5 indicate a compound with strong inhibitory activity. In addition, C4' hydroxylation and either C3' hydroxylation or methylation of Ring B improves inhibitory potential. The hydrophobic basic structure of flavones aids in binding to the active site of aromatase as does hydrogen bond donor/acceptor areas close to C4' of Ring B and C5 and C7 of Ring A (64). It is also suggested that prenylation of C8 also significantly increases inhibitory activity as in the case of 4', 5, 7-trihydroxy-8-prenylnaringenin (61). In general, it can be concluded that flavonoids are the strongest group of phytochemical aromatase inhibitors.

4.2. Flavanols

Studies into the anti-aromatase activity of flavanols show that, while the tea catechins show activity, catechin itself is not an AI. Many of the studies were done in the human placental microsome assay (21, 65), however, preadipocyte and adrenocortical cell culture systems were also utilized (53, 58). In both cell culture systems and the placental microsomes, catechin and epicatechin were found to have no anti-aromatase activity (53, 58, 65). Interestingly, catechin gallate, epicgallocatechin,

Table 2. Reported inhibition of aromatase by flavanols

Compound	Assay	IC_{50} (μM)	Reference
Catechin	Preadipocyte cell	NE	(53)
Catechin	Adrenocortical carcinoma cell	NE	(58)
Catechin gallate	Placental microsome	55	(65)
Epicatechin	Adrenocortical carcinoma cell	NE	(58)
Epigallocatechin	Placental microsome	100	(65)
Epigallocatechin gallate	Placental microsome	60	(65)
Epigallocatechin gallate	Placental microsome	13.79	(21)
Gallocatechin gallate	Placental microsome	15	(65)
Polyphenone-60	Placental microsome	28l	(65)
Theaflavin	Placental microsome	4.17	(21)
Theaflavin 3,3'-digallate	Placental microsome	3.45	(21)
Theaflavin 3-gallate	Placental microsome	3.23	(21)

NE=no effect

epigallocatechin gallate and gallocatechin gallate exhibited strong anti-aromatase activity (21, 65). In addition, the oligomeric tea flavonols theaflavin, theaflavin 3,3'-digallate and theaflavin 3-gallate (21) and a water extract of green tea catechins (called polyphenone-60) were also strong inhibitors (65). Kinetic studies showed that the theaflavins exhibit mixed inhibition of the aromatase enzyme. In this study, the theaflavins were all more potent than EGCG in the placental microsome assay, indicating that these compounds may be more effective AIs than tea catechins (21). In addition, the mixed compound polyphenone-60 was an extremely strong inhibitor, illustrating the benefit of phytochemical combinations. The IC_{50} values of the flavanols from reported studies are listed in Table 2.

4.3. Flavanones

The flavanones that have been tested for anti-aromatase activity consist of flavanone, 4'-hydroxyflavanone, 7-methoxyflavanone, hesperetin and naringenin. In all studies, flavanone was shown to be an inhibitor of aromatase (51, 52, 66) as were 4'-hydroxyflavanone (52) and hepseritin (54). In contrast, 7-methoxyflavone did not exhibit any inhibitory action. The observed anti-aromatase activity of naringenin varied based on the method used. Le Bail and Edmunds *et al* both reported that naringenin showed significant anti-aromatase activity in placental microsomes (55, 67) and co-culture experiments (59) whereas it showed weak activity in adrenocortical cells (58). These results are likely due to the difference in assay method, with the in-cell assay requiring higher concentrations to elicit a response based on cellular uptake and metabolism. Overall, flavanones can also be considered a group of compounds with significant anti-aromatase activity. The IC_{50} values of the flavanones from reported studies are listed in Table 3.

4.4. Flavonols

In fruits, quercetin glycosides are the predominant flavonols whereas in vegetables, quercetin is the principal flavonol, with glycosides of kaempferol also present (68). Quercetin showed significant anti-aromatase activity (51) in the placental microsome assay, however, it exhibited either a mild or no effect in live cell assays utilizing adrenocortical carcinoma cells (58), preadipocyte cells (34), or in co-culture experiments (59). This is again likely due to the added complication of cell uptake and metabolism in the in-cell assays. *In vivo* experiments are necessary to determine the efficacy of these compounds in a whole body system. The flavonols fisetin and morin were also found to have no effect, whereas kaempferol exhibited mild anti-aromatase activity (IC_{50} of 61 μM) in the preadipocyte assay system (34). The IC_{50} values of the flavonols from reported studies are listed in Table 4. Flavonols can be considered weak AIs, however, it is thought that the benzopyranone ring structure may be a suitable template on which to base the design of synthetic AI drugs (69).

4.5. Isoflavones

Isoflavones are a large group of phenolic compounds that have been shown to have both estrogenic and anti-estrogenic effects *in vitro* and *in vivo*. With regard to inhibition of the aromatase enzyme, isoflavones have been shown to exhibit limited anti-aromatase activity. Daidzein, equol, genistein and 0-desmethylangolensin all had no effect on aromatase activity in both microsomal and in-cell assays (53, 55, 58, 66, 67, 70). The one assay where genistein showed significant anti-aromatase activity was in the co-culture experiments with MCF-7 and fibroblast cells (59), illustrating the possible variability in results based on the assay type or the complication of metabolism by the cell lines in the assay.

A combination mixture of genistein, daidzein and the flavone biochanin A was shown to significantly reduce aromatase mRNA expression in primary cultures of human granulosa luteal cells (71). This study is promising because it demonstrates that despite a lack of activity when the compound is in isolation, the combination of phytochemicals naturally found in the diet can work together to achieve a positive response. The IC_{50} values of the isoflavones from reported studies are listed in Table 5.

4.6. Coumarins

Coumestrol and several coumarin derivatives have demonstrated anti-aromatase activity when tested in the placental microsome assay (34, 55, 72). In one major study, twenty-one coumarin derivatives were tested for anti-aromatase activity in the placental microsome assay and six of these were found to be active: 4-benzyl-3-(4'-chlorophenyl)-7-hydroxycoumarin, 4-benzyl-7-methoxy-3-phenylcoumarin, 3-(4/-bromohenyl)-7-hydroxy-phenylcoumarin, 6-hydroxy-3-(4'-methoxyphenyl)-4-methylcoumarin and 4-benzyl-7-hydroxy-3-phenylcoumarin. Three of these compounds

Table 3. Reported inhibition of aromatase by flavanones

Compound	Assay	IC_{50} (μM)	Reference
4'-hydroxyflavanone	Placental microsome	10	(52)
7-hydroxyflavanone	Adrenocortical carcinoma cel	65	(58)
7-methoxyflavanone	Adrenocortical carcinoma cell	NE	(58)
Flavanone	Placental microsome	8	(52)
Flavanone	Placental microsome	8	(51)
Flavanone	Placental microsome	250	(66)
Hesperitin	Placental microsome	3.31[1]	(54)
Naringenin	Placental microsome	9.2	(55)
Naringenin	Adrenocortical carcinoma cell	85	(58)
Naringenin	Fibroblast/MCF-7 co-culture	2.2	(59)
Naringenin	Placental microsome	2.9	(63)
Naringenin	CHO cell expression	5.1[2]	(56)

NE=no effect, [1]molarity calculated from IC_{50} given in research paper in μg/ml. [2]Ki value

Table 4. Reported inhibition of aromatase by flavonols

Compound	Assay	IC_{50} (μM)	Reference
Fisetin	Preadipocyte cell	NE	(34)
Kaempferol	Preadipocyte cell	61	(34)
Morin	Preadipocyte cell	NE	(34)
Quercetin	Placental microsome	12	(51)
Quercetin	Adrenocortical carcinoma cell	NE	(58)
Quercetin	Preadipocyte cell	NE	(34)
Quercetin	Fibroblast/MCF-7 co-culture	30	(59)

NE = no effect

Table 5. Reported inhibition of aromatase by isoflavones

Compound	Assay	IC_{50} (μM)	Reference
Biochanin A	CHO cell expression	12	(56)
Daidzein	Placental microsome	<1000	(70)
Daidzein	Preadipocyte cell	NE	(53)
Daidzein	Placental microsome	NE	(55)
Equol	Placental microsome	150	(70)
Equol	Preadipocyte cell	NE	(53)
Equol	Placental microsome	850	(66)
Genistein	Preadipocyte cell	NE	(53)
Genistein	CHO cell expression	123[1]	(56)
Genistein	Placental microsome	NE	(55)
Genistein	Adrenocortical carcinoma cell	NE	(58)
Genistein	Fibroblast/MCF-7 co-culture	3.6	(59)
Methylequol	Placental microsome	20	(70)
0-Desmethylangolensin	Placental microsome	160	(70)
0-Desmethylangolensin	Preadipocyte cell	NE	(34)

NE=no effect. [1]Ki value

were extremely active and exhibited IC_{50} values of 1 μM or below, kinetic analysis identified them as competitive inhibitors of aromatase (72). Hong *et al*, recently demonstrated that coumestrol was a weak AI (73). In addition, the authors identified S478 and H480 in the active site of aromatase as residues that are involved in coumestrol binding. The authors also produced a model for the docking position of coumestrol into the active site of aromatase, based on the hypothesis that rings A and C of coumestrol mimic rings A and B of estradiol. Due to the small size of coumestrol, it is thought that this molecule can enter deeply into the active site, resulting in close contact with the S478 and H480 residues. In general, planar flavones such as coumestrol have a low affinity for binding to aromatase, which supports the observation that phytoestrogens bind aromatase with a lower affinity than the natural substrates (73).

The interaction of the coumarin derivatives with aromatase was tested using four different aromatase constructs with mutations in the active site of the enzyme. In this study, the inhibitory activity of 4-benzyl-3-(4'-chlorophenyl)-7-methoxycoumarin was significantly decreased in these mutants when compared to the wild type aromatase. These studies confirm that the coumarins interact with the active site of aromatase, as Glu-302, Thr-310, Ser-478 and his-480 (mutated in these studies) have all been shown to be important to substrate binding (74). The relationship between the structure of 4- benzyl-3-(4'-chlorophenyl)-7-methoxycoumarin and its activity against the enzyme was then modeled via computer. From this modeling, it can be seen that the 7-methoxyl, 3-(4'-cholrophenoyl) and 4-benzyl groups are integral to the anti-aromatase activity of the coumarins (72).

In contrast, a recent paper which modeled the interaction of coumarins with the active site of aromatase found that the coumarins in the panel that these authors investigated were very weak AIs. The authors suggest that the benzopyrone hydrophobic scaffold of the coumarins is too short to make a strong interaction with the active site of aromatase (64). However, the difference between these

Table 6. Reported inhibition of aromatase by coumarins

Compound	Assay	IC_{50} (μM)	Reference
3-(4'-bromophenyl)-7-hydroxy-4-phenylcoumarin	Placental microsome	6	(72)
4-benzyl-3-(4'-chlorophenyl)-7-hydroxycoumarin	Placental microsome	0.3	(72)
4-benzyl-3-(4'-chlorophenyl)-7-methoxycoumarin	Placental microsome	0.08	(72)
4-benzyl-7-methoxy-3-phenylcoumarin	Placental microsome	1	(72)
6-hydroxy-3-(4'-methoxyphenyl)-4-methylcoumarin	Placental microsome	16	(72)
Coumestrol	Placental microsome	25	(55)
Coumestrol	Preadipocyte cell	17	(34)
Coumestrol	CHO cell expression	4[1]	(73)

NE=no effect. [1]Ki value

two studies likely lies in the dissimilarity of the specific coumarins tested. The compounds in the earlier study were larger and therefore would not be limited by their size with respect to interaction with the enzyme. Additionally, as can be seen with any of the compounds tested for anti-aromatase activity, minor changes to the structure can drastically change their activity. The IC_{50} values of the coumarins from reported studies are listed in Table 6.

5. NON-FLAVONOID PHENOLIC COMPOUNDS

5.1. Lignans

Lignans are a major class of phytoestrogens produced by the intestinal bacteria from precursors found in a variety of plants. Studies done by Adlercreutz *et al* have shown that the urinary excretion of lignans and isoflavones in breast cancer patients is significantly lower than that of healthy controls (75). Therefore, they hypothesized that low dietary intake of these phytochemicals could be a risk factor for breast cancer. This group tested a panel of lignans using the human placental microsome assay. Of the phytochemicals tested, the authors found enterolactone to be a moderate AI (IC_{50} of 14 μM) and methylequol and enterodiol exhibited less activity (IC_{50} of 20 and 30 μM, respectively). They also tested three synthetic lignans and found 3'-demethoxymatairesinol was a weak inhibitor, but both 4,4'-dihydroxyenterolactone and 4,4'-enteraolacone exhibited significant inhibitory activity. Kinetic analysis revealed that enterolactone is a competitive inhibitor, whereas the spectroscopic analysis suggested that enterolactone displaces the substrate, androstenedione, from the active site of aromatase. The authors hypothesized that enterolactone either binds directly to the substrate region of the active site or close to it, causing a conformational change (70).

Various lignan compounds have also been tested for anti-aromatase activity in a preadipose cell culture system. Enterolactone and its precursors 3'-demethoxy-3O-demethylmatairesinol (DMDM) and didemethoxymatairesinol (DDMM) were analyzed along with enterodiol and its precursors O-demethylsecoisolariciresinol (ODSI) and demethoxysecoisolariciresionl (DMSI) and didemethylsecoisolariciresionl (DDSI). The results of this study showed that enterolactone, DMDM and DDMM possess mild anti-aromatase activity (IC_{50} of 61, 74, 84, and 60 μM, respectively). The comparison of the level of activity was made to the control inhibitor, aminoglutethimide, which had an IC_{50} of 5 μM in this assay system (34). Kinetic analysis indicated that all of the active compounds were competitive inhibitors, which is in agreement with previous studies of phytochemicals and suggests a common mechanism of action in natural aromatase inhibitors. In agreement with the Adlercreutz study, this study confirmed that enterodiol has a lower anti-aromatase activity than enterolactone.

The authors of this study also made observations on the structure of the active compounds and noted that all of the active inhibitors contained a C-7 hydroxyl group. They further noted that a hydroxyl group at C-3 correlated with decreased activity and therefore this would be an undesirable structural component for natural aromatase inhibitors. In addition, since a hydroxyl group at C-5 was scattered among the inhibitors and non-inhibitors, that this hydroxylation would be unimportant to the anti-aromatase activity of the compound (34). The IC_{50} values of the lignans from reported studies are listed in Table 7.

5.2 Resveratrol

Resveratrol is non-flavonoid phenolic compound belonging to the stilbene family. It is a phytoalexin produced in a variety of plants but mainly found in grapes and berries. Due to its presence in the skins of red grapes, resveratrol is also found in red wine. A recent study investigated the anti-aromatase activity of resveratrol and found that the activity of recombinant aromatase was significantly inhibited by resveratrol in a dose-dependent manner. In JEG-3 placental cells, resveratrol significantly inhibited aromatase activity with an IC50 value of 17 μM. In addition, reporter gene assays showed significant inhibition of the transactivation of aromatase promoter Ia (the promoter primarily responsible for aromatase expression in placental cells) by resveratrol (76).

Further study by this group also showed that treatment of SKBR-3 aromatase expressing cells with 12.5 micro-M significantly decreased aromatase mRNA expression and 50 μM resveratrol completely abolished it. In addition, after 24 hours of treatment, resveratrol significantly decreased aromatase activity with an IC_{50} of 20 μM and inhibited estradiol-induced aromatase protein expression. Concentrations of resveratrol at 6.3 μM and greater significantly repressed CYP19 promoter I.1 activity. Resveratrol was shown not to compete for binding to the ER or decrease ERE-mediated transactivation. Interestingly, estrogen-induced extracellular regulated kinase 1/2 (ERK1/2) activity was decreased by 25 μM of resveratrol showing an effect on non-genomic cell signaling pathways in breast cancer cells (77).

Table 7. Reported inhibition of aromatase by lignans

Compound	Assay	IC_{50} (μM)	Reference
Enterodiol	Placental microsome	30	(70)
Enterodiol	Preadipocyte cell	>100	(34)
Enterolactone	Placental microsome	14	(70)
Enterolactone	Preadipocyte cell	74	(34)
Nordihydroguaiaretic acid	Placental microsome	11	(70)
3'-demethoxymataires inol	Placental microsome	37	(70)
4,4'-dihydroxyenterolactone	Placental microsome	6	(70)
4,4'-enterolactone	Palcental microsome	15	(70)

NE=no effect

Table 8. Reported inhibition of aromatase by resveratrol

Compound	Assay	IC_{50} (μM)	Reference
Resveratrol	SKBR-3 cell	20	(77)
Resveratrol	JEG cell Recombinant aromatase	17 40	(76)
Resveratrol	MCF-7aro cell	25	(78)
Resveratrol	SKBR-3 cell	43	(78)

NE=no effect

Resveratrol was also shown to inhibit aromatase activity in MCF-7aro aromatase over-expressing cells with an IC_{50} value of 25 μM. Kinetic analysis revealed a mixed type of inhibition. In this same cell line, 10 μM treatment with resveratrol significantly reduced testosterone-induced cell proliferation and 50 μM completely inhibited it. In SKBR-3 cells, 50 μM of resveratrol decreased CYP19 mRNA and protein expression, promoter I.3/II-luciferase activity and in-cell aromatase activity with an IC_{50} of 25 μM (78). These studies suggest that resveratrol could inhibit estrogen biosynthesis both through its action on aromatase expression and activity and exhibits further anti-cancer effects through inhibition of cell survival signaling pathways such as ERK1/2. The IC_{50} values of resveratrol from reported studies are listed in Table 8.

5.3 Alkaloids

5.3.1. Nicotene and anabasines

Epidemiologic studies have associated cigarette smoking with early menopause and decreased estrogen levels in the urine of women (79-81), suggesting that the compounds in tobacco and/or tobacco smoke can have an effect on the endocrine system. An interesting observation occurred by chance when it was discovered that cigarette smoke can interfere with the microsomal aromatase assay. Therefore, the effects of tobacco leaf and smoke extracts on aromatase activity were investigated. Initial experiments showed the basic fraction of cigarette smoke to be the most potent anti-aromatase compound, although water extracts of cigarette smoke also inhibited granulosa cell aromatase. It was found that 90% of the inhibitory action of the water extract was contained in the low molecular weight fraction, where nicotine and anabasine would be found. These two purified constituents also inhibited aromatase (82). In addition, in both choriocarcinoma cell cultures and placental microsomes, nicotine, cotinine and anabasine significantly inhibited aromatase on a competitive manner with respect to the substrate (83).

Investigations into extracts of tobacco leaf and smoke extracts have shown that the basic fraction of cigarette smoke contains the highest anti-aromatase activity. Purification of this fraction led to the isolation of N-n-octanoylnornicotine (84). A number of other acylated normicotines and anabasines were synthesized and tested for anti-aromatase activity. The authors discovered that the acylated nornicotines had significantly higher activity than the parent compounds nicotine and anabasine. The activity of the compounds was related to the length of the acyl carbon chain, with C-11 showing the most activity. Administration of this compound to rats inhibited NMU-induced breast tumor growth, illustrating activity in vivo (84).

Kinetic analysis showed that N-n-octanoylnornicotine inhibits placental microsomal aromatase in a competitive manner with a Ki of 0.65 □M. In addition, other acyl-derivatives (N-n-Decanoylnornictoine and N-(4-hydroxyundecanoyl) nornicotine) were also inhibitors with a Ki of 0.86 and 0.24 □M, respectively. The same group utilized aromatase expressing cell lines and found that both N-n-octanoylnornicotine and N-(4-hydroxyundecanoyl) anabasine showed anti-aromatase activity in this assay (85). In addition, the kinetics of the inhibition by N-(4-hydroxyundecanoyl) anabasine was evaluated in microsomes prepared from aromatase expressing cells or breast tumor tissue and the inhibition of aromatase was also found to be competitive, which is in agreement with studies carried out by other investigators (36). The IC_{50} values of the alkaloids from reported studies are listed in Table 9. Although smoking has not been shown to have a protective effect on breast cancer incidence, these studies show that compounds found in tobacco and cigarette smoke may be isolated and utilized to inhibit estrogen biosynthesis.

6. PERSPECTIVE

The expected direct outcome of chemoprevention through aromatase inhibition is the maintenance of low levels of estrogen in the breast and surrounding adipose tissue. Theoretically, this would lead to a decreased incidence of breast cancer occurrence/recurrence as clinical trails have shown an increase in disease-free survival with third generation AI treatment. In addition, the ATAC, BIG 1-98, MA 17 and IES clinical trials have demonstrated a

Table 9. Reported inhibition of aromatase by alkaloids

Compound	Assay	IC_{50} (μM)	Reference
N-(4-hydroxyundecanoyl) anabasine	Human placenta	30	(36)
	SKBR-3 cell	2	
	MDA-MB-231 cell (non-induced)	20	
	MDA-MB-231 cell (DEX-induced)	15	
	MDA-MB-231 cell (cAMP-induced)	11	
	Placental microsome	5	
N-n-octanoylnornicotine	Human placenta	360	(36)
	SKBR-3 cell	450	
	MDA-MB-231 cell (non-induced)	310	
	MDA-MB-231 cell (DEX-induced)	200	
	MDA-MB-231 cell (cAMP-induced)	150	

NE=no effect

significant decrease in the cases of contralateral breast cancer in the AI treated versus Tamoxifen treated patients (9-12). Because aromatase is an enzyme that is expressed in tissues other than the breast, natural AIs could also inhibit the development of other estrogen-related cancers such as endometrial cancer. Aromatase inhibition by isolated natural products utilized in supplement form is a viable chemopreventive strategy; however, not only the efficacy of the compounds must be evaluated *in vivo* but the toxicity as well. One advantage of the intake of whole foods known to be rich in anti-aromatase phytochemicals is the potential low toxicity. The fact that a specific ingredient or a number of different foods can be incorporated into the diet speaks to the idea that long-term exposure to low doses of a variety of phytochemicals can be efficacious while avoiding serious side effects. We do know from the *in vivo* studies with grape seed extract and white button mushroom that these whole plant extracts are orally active, meaning they inhibited estrogen-induced breast tumor growth in the mouse xenograft model.

In a majority of the published reports, the strength of an inhibitor is measured by its IC_{50} value. While comparing these values between publications may be misleading due to variations in assay method and the laboratory, this measure can be useful when the studies are put together and relative potency can be evaluated. With respect to the relative potency of the natural compounds, it is clear that the structure of the flavones makes them a potent group of AIs. In comparison, the isoflavones are not as effective. Flavones bind to the active site of aromatase in such a way that the A and C rings mimic the D and C rings of the substrate. Alternatively, the isoflavones contain a 4'-hydroxy group in the equivalent C-6 position in the flavones, decreasing enzyme binding. In addition, the isoflavones contain a 4'-hydroxyphenyl group at the C-3 position which also inhibits enzyme binding (74). Based on the current findings, the activity of the flavanols is variable, with the tea catechins CG, EGC and EGCG showing significant activity whereas catechin itself has limited activity. In general, the flavanones are active inhibitors where the flavonols have weak to no inhibitory activity. With the coumarins, it is apparent that the size of the coumarin compound determines its potency, with the smaller compounds showing no activity. Lignans appear to be moderate to weak inhibitors of aromatase whereas the alkaloids nicotine, cotinine and anabasine show significant activity with the acylated compounds more active than the basic compounds. In general, how well the compound mimics the substrates for aromatase (testosterone and androstenedione) determines its ability to bind and directly inhibit the enzyme.

Although this review focuses on natural AIs, a number of laboratories are currently investigating synthetic AIs that are based on the structures of natural compounds. The goal with this type of research is to improve upon the natural compounds by altering their chemical structure based on structural characteristics that are known to increase the effectiveness of a compound. This is a viable strategy for the design of new AIs; however these compounds were not covered in detail in this review as they are not classified as "natural compounds" due to their synthetic nature.

Much of the research into natural AIs has evaluated the ability of natural products to inhibit the aromatase enzyme, however it is equally important to search for phytochemicals that can selectively suppress aromatase expression in breast cancer tissue through the suppression of the aromatase promoters I.3/II, which are the most active promoters in breast cancer tissues (86-88). Our laboratory has initiated this type of research with our studies into grape seed extract (41). In addition, the stability and bioavailability of potential aromatase inhibitory phytochemicals is also an area that is under-studied. *In vivo* bioavailability studies are critical for the application of phytochemicals in chemoprevention. Furthermore, the phytochemicals must be orally active, as was shown with grape seed (41) and mushroom extract (37), to be of any real benefit. Phytochemicals that may be potent inhibitors *in vitro* may be ineffective *in vivo* and therefore useless as chemopreventive agents. Currently, a Phase I chemoprevention clinical trial investigating the effects of grape seed extract in post-menopausal women is underway at the City of Hope and Phase I trials of mushroom powder in both breast and prostate cancer survivors have been initiated (http://clinicaltrials.coh.org). More trials of this type are needed to determine the safety and efficacy of natural chemopreventive agents.

The long term effects of aromatase inhibition in healthy individuals are currently unknown. In addition, the consequences of inhibition in different stages of life have yet to be determined. It is possible that AIs may affect a developing fetus or a child differently than a teen or an adult female. For example, studies in aromatase knockout mice show that the mice become obese by 3 months of age

(89), suggesting one consequence of estrogen deficiency in young girls. Further *in vitro* and *in vivo* study is needed to more definitively clarify the appropriate timing and chemopreventive applications for the use of specific classes of phytochemicals. However, a diet rich in whole foods containing phytochemicals with anti-aromatase activity is unlikely to have adverse effects at the low levels in which they are present.

A more comprehensive knowledge of the biologic effects of natural aromatase inhibitors *in vivo* is needed to aid in the design of realistic prevention strategies. Although it is necessary to identify the active compounds in individual plants, natural products are extremely complex in their chemical makeup. Within the active crude fractions of a specific plant, many separate compounds likely exist that play a role in that activity. In addition, the chemopreventive activity of a food in total may be due to different compounds with discrete mechanisms beyond the inhibition of aromatase, such as ER antagonism or inhibition of cellular signaling. Therefore, synergy is more than likely the key to successful chemoprevention with natural products. Overall, the above-reviewed studies represent a large body of work that taken together, suggest a very definite role for phytochemicals in the inhibition of breast cancer through aromatase inhibition.

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