

Aromatase Inhibition: Translation into a Successful Therapeutic Approach

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Abstract The development of the novel third-generation aromatase inhibitors and inactivators for breast cancer treatment is one of the most successful contemporary achievements in cancer therapy. Parallel to studies evaluating toxicity and clinical efficacy in metastatic disease, the endocrine effects of multiple compounds were evaluated, leading to the identification of the highly potent third-generation aromatase inhibitors based on estrogen deprivation and aromatase inhibition *in vivo*. Thus, translational studies have been of vital importance identifying the unique characteristics of these compounds. Whereas first- and second-generation aromatase inhibitors inhibit estrogen synthesis *in vivo* by up to 90%, the third-generation compounds anastrozole, exemestane, and letrozole were found to cause $\geq 98\%$ aromatase inhibition. This article summarizes and discusses the “translational research” that provided the background for the implementation of the third-generation aromatase inhibitors and inactivators into large clinical trials. The need for future translational research exploiting the mechanisms of resistance to these compounds for future improvement of endocrine therapy is emphasized.

The development of contemporary endocrine treatment for breast cancer is one of the most remarkable stories in cancer therapy. Initiated more than a century ago by Beatson's oophorectomy (1), followed by other pioneering discoveries like the therapeutic potential of ablative strategies (adrenalectomy or hypophysectomy) in postmenopausal (2, 3) and the beneficial effects of different additive therapies (refs. 4–7; Table 1), endocrine treatment of breast cancer was established a long time before implementation of cytotoxic therapy (8–14). Currently, we are facing a major novel achievement: the successful development of the third-generation aromatase inhibitors and inactivators in early breast cancer.

Traditionally, endocrine therapy has been developed through interplay between clinical observations, endocrine evaluations *in vivo*, and laboratory experiments. The initial clinical discoveries with respect to oophorectomy, additive therapy with estrogens, and adrenalectomy were empirical observations at a time when knowledge about endocrinology in general and with respect to breast cancer in particular was poor. At this stage, the clinical observation of the effects of ablative as well as additive treatment added key information to our understanding of the endocrine biology of breast cancer. As such, the development of endocrine therapy remains a pioneering example of successful translational research, transferring knowledge between basic research and the clinic in an

interactive two-way process (15, 16). As may be seen, this applies to the successful development of third-generation aromatase inhibitors and inactivators in particular.

The discovery of the estrogen receptor and, subsequently, the finding three decades ago that its expression was a requisite for response to endocrine therapy (17, 18) gave us a predictive factor, something yet to be identified for chemotherapy. Although far from perfect (we still lack knowledge why so many tumors harboring the estrogen receptor- α do not respond to endocrine therapy), the lack of response among tumors with negative receptor status means that a substantial number of patients may avoid an ineffective therapy. The functional role of the ligand-receptor mechanism is further underlined by the additional predictive value of the progesterone receptor, known to be a marker of a functional estradiol (E_2)-receptor ligand interaction (19). Whether its predictive value differs with respect to treatment with tamoxifen and aromatase inhibitors (20) remains to be confirmed in additional studies.

The list of unexpected clinical observations leading to a “conceptual breakthrough,” thus, “translating the clinic back to the laboratory,” is extensive. The introduction of the first aromatase inhibitor in the clinic was based on a conceptual misunderstanding. The therapeutic effect of surgical adrenalectomy initiated attempts to develop medical adrenalectomies by use of corticosteroids and, later, an adrenotoxic antiepileptic compound named aminoglutethimide (13, 21–23). Whereas treatment with corticosteroids was only partly successful (24, 25), the clinical efficacy of aminoglutethimide triggered endocrine studies revealing the compound to be a potent inhibitor of aromatization, albeit with additional effects on adrenal steroid synthesis (26, 27).

The observation that the response to hormone ablation was (at least) as good in postmenopausal patients undergoing adrenalectomy as for premenopausal patients treated with ovarian ablation and, later, the finding that aminoglutethimide may

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Table 1. Development of endocrine therapy

First published	Author	Type of therapy	References
1896	Beatson	Oophorectomy	(1)
1922	DeCourmelles	Ovarian irradiation	(9)
1939	Ulrich	Androgens	(10)
1944	Haddow et al.	Synthetic estrogens	(5)
1952	Douglas	Pituitary irradiation	(11)
1953	Huggins et al.	Adrenalectomy	(12)
1953	Luft et al.	Hypophysectomy	(3)
1956	Taylor et al.	Cortical steroids	(6)
1971	Cole	Anti-estrogen	(14)
1973	Schwarzel et al.	Aromatase inhibition	(22)

work even in women expressing supra-low plasma estrogen levels due to a previous adrenalectomy or hypophysectomy (28) revealed the (still puzzling) observation that endogenous estrogens may stimulate the growth of hormone-sensitive tumors more or less independent of plasma hormone levels, suggesting the antitumor effects of hormone suppression may be due to relative suppression and not absolute hormone levels (29).

Another clinical observation translating into an improved conceptual understanding relates to the clinical experiences made during treatment of breast cancer patients with androgens. Whereas androgens have antitumor effects but were abandoned from breast cancer therapy due to their side effects (7), the discovery of their antitumor effects may add important information to our understanding of the puzzling lack of cross-resistance between aromatase inhibitors and inactivators (see later). Finally, recent clinical observations with use of tamoxifen in combination with luteinizing hormone-releasing hormone analogues in premenopausal (30) and tamoxifen in combination with aromatase inhibitors in postmenopausal (31) have added to our understanding of the estrogen agonistic/antagonistic effects of tamoxifen in relation to estrogen suppression.

An important issue with respect to most forms of therapy is the search for biochemical surrogate markers predicting *in vivo* efficacy. In contrast to other forms of endocrine treatment, like use of antiestrogens, for which biochemical surrogate variables remain an issue of debate (32, 33), or chemotherapy, for which dose-finding is based on a combined assessment of preclinical efficacy and *in vivo* toxicity, the effect of an aromatase inhibitor on its therapeutic target, the peripheral aromatase enzyme, can be determined *in vivo* (34). Whereas aromatase inhibitors may be characterized based on *in vitro* experiments, their potency *in vivo* will depend on additional factors, including plasma half-life and tissue penetration. Thus, whereas fadrozole was found superior compared with letrozole *in vitro* (35, 36), letrozole was found to be much more potent *in vivo* (37, 38).

This review will focus on the translational work leading to the successful development of novel aromatase inhibitors. The issues of endocrine assessment will be discussed in detail, putting emphasis on methodologic limitations, as these are critical to the interpretation of our findings. The fact that aromatase inhibitors may improve relapse-free and,

most likely, overall survival compared with tamoxifen (31, 39, 40), something few had believed a decade ago, underlines the potential for improvement in this therapeutic area.

Development of Aromatase Inhibition as a Therapeutic Strategy in Breast Cancer

The first aromatase inhibitor for clinical use, an adrenotoxic antiepileptic named aminoglutethimide, was introduced in an attempt to induce a medical adrenalectomy (13, 41). However, over the next decade, Santen et al. (27, 42), in a series of elegant experiments, revealed plasma dehydroepiandrosterone sulfate to be suppressed, but plasma androstenedione sustained or elevated depending on glucocorticoid replacement. Despite this, they found plasma estrogens to be profoundly suppressed. Inspired by the work of Thompson and Siiteri (23), who found aminoglutethimide to inhibit *in vitro* aromatization in 1974, they evaluated and found aminoglutethimide to inhibit *in vivo* aromatization in postmenopausal women (26; see ref. 43 for a detailed discussion of the early clinical development and endocrine effects of aminoglutethimide). The clinical development of aminoglutethimide as an aromatase inhibitor was then pursued. However, due to the side effects of the nonselective compound aminoglutethimide, many attempts were made to find more selective drugs that were better tolerated.

At the same time, Brodie and colleagues identified a panel of steroidal compounds inhibiting the aromatase enzyme in preclinical models (44, 45). The first approach was developing substrate analogues, derivatives of androstenedione (the major substrate of aromatase) or testosterone.

Following the results of Santen and colleagues, two independent events, one endocrine study and one pilot study of the antitumor effects of a compound, confirmed the principle of aromatase inhibition, independent of adrenal enzyme inhibition, as a therapeutic strategy in breast cancer. The endocrine study related to the compound named testololactone. This weak androgen agonist had been evaluated for breast cancer therapy in the 1970s and found to cause antitumor effects (46) albeit with a significant toxicity. Subsequently, Barone et al. (47) in 1979 found testololactone to inhibit peripheral aromatization by about 90%, confirming the compound to be a "steroidal" aromatase inhibitor, albeit with intrinsic androgenic effects. The "clinical event" was the pilot study reporting 4-hydroxyandrostenedione (today known as formestane), one of the compounds developed by the Brodie team, to cause antitumor effects in patients suffering from metastatic breast cancer (48). As this compound has no effects on adrenal hormone synthesis nor does it express any androgenic activity following parenteral administration (49, 50), this small study provided the "proof of principle" revealing a selective aromatase inhibitor to be an active antitumor compound.

Aromatase Inhibitors and Inactivators

There are two classes of aromatase inhibitors; the nonsteroidal, or type II compounds, and the steroidal derivatives (type I), currently coined aromatase inactivators (see explanation in this section). Considering the nonsteroidal compounds, these drugs are either phenobarbitone derivatives (like aminoglutethimide) or belong to the imidazole/triazole class (Fig. 1). The steroidal

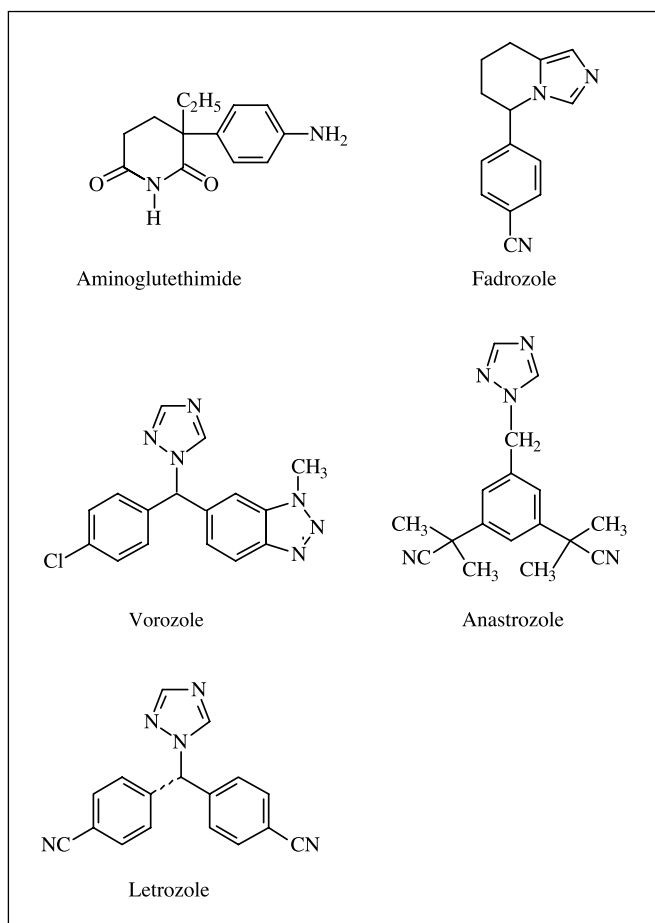


Fig. 1. Chemical structures of some nonsteroidal aromatase inhibitors.

compounds are derivatives of the aromatase substrate, androstenedione (Fig. 2).

Based on *in vitro* experiments, the nonsteroidal compounds are characterized by reversible binding to the P450 part of the aromatase enzyme. In contrast, the two steroidal compounds used for breast cancer therapy (4-OH-androstenedione and exemestane) both bind irreversibly to or near by the substrate-binding pocket due to covalent bonds causing aromatase inactivation (51–54). Through this mechanism the enzyme is irreversibly inactivated (also called mechanism based or suicide inhibition; ref. 55). The potential importance of these differences (56), in particular with respect to the well-confirmed lack of complete cross-resistance between aromatase inhibitors and inactivators, has been an issue of debate. So has the observation that the steroidal compounds express slight androgenic effects as detected by a dose-dependent suppression of plasma sex hormone binding globuline following oral but not parenteral administration (57, 58). Whereas the important issue of lack of cross-resistance is addressed later in this article, it should be mentioned that this observation, similar to the discovery of aminoglutethimide as an aromatase inhibitor, was made for the first time in a clinical trial (59) for which the hypothesis (that formestane should be a more potent inhibitor compared with aminoglutethimide) was incorrect, but the therapeutic result turned out to be positive, triggering the postulation of an alternative hypothesis.

There are three types of methods by which the biochemical efficacy of an aromatase inhibitor may be assessed: *in vitro* measurements, evaluation in animal models, and *in vivo* assessment of endocrine efficacy in humans. Whereas the two first issues will be covered here briefly and the reader referred to other contemporary reviews, this article will review the subject of endocrine assessment in humans in detail.

In vitro Assessment

In general, *in vitro* assessment of aromatase inhibition is conducted using placental or ovarian aromatase as test substrate (60). Notably, whereas aromatase expression may be regulated by different promoters in different tissues (refs. 61, 62; see later), only one aromatase gene, coding a single protein, is detected in human tissues (63). The subject of *in vitro* assessment of aromatase inhibitors has been reviewed by others (64, 65); only a few issues will be highlighted here.

Whereas *in vitro* data may outline the potency of individual compounds suggesting which may be chosen for clinical development, the importance of *in vivo* assessment of endocrine effects is illustrated by the comparison between fadrozole and letrozole, revealing fadrozole to be more potent *in vitro* (35, 36), whereas letrozole was superior *in vivo* (37, 38). Whether the discrepancy between *in vitro* and *in vivo* findings may be related to differences in pharmacokinetic disposition (66–68) alone or other factors may contribute is not known.

Animal Models

The animal model most frequently used to assess endocrine effects of aromatase inhibitors is the ovariectomized nude mouse inoculated with MCF-7 cells transfected with the human aromatase gene (MCF-7_{CA}) and treated with

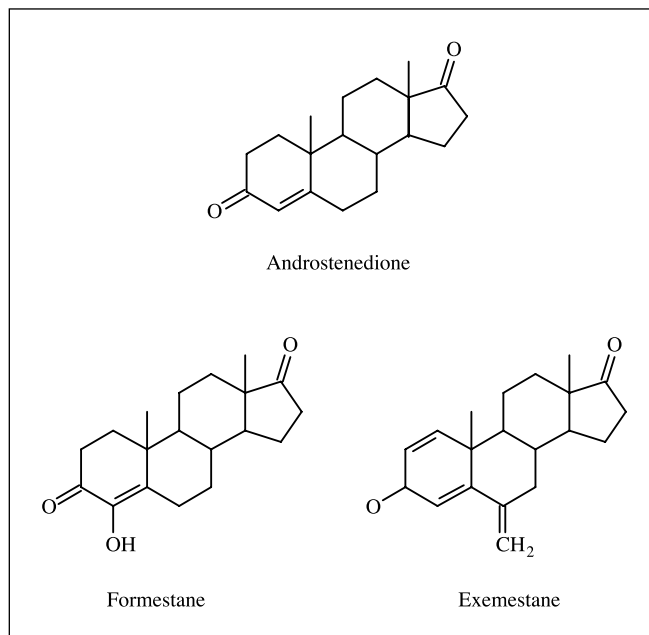


Fig. 2. Chemical structures of androstenedione and some steroidal aromatase inactivators.

androgens (postmenopausal model; ref. 64, 69). Whereas results achieved with this model have been interpreted in favor of intratumor estrogen synthesis versus systemic delivery (69, 70), there is evidence that estrogens synthesized by the MCF-7 xenografts in this model may contribute to circulating hormone levels (71). However, the relevance of this source to circulating estrogens in breast cancer patients remains uncertain.

Recently, insights into the effects of estrogens on different target tissues have been generated by animal models using targeted disruption of key genes (knockout models). This group comprises the aromatase-knockout mouse (ref. 72; ArKO mouse), the ERKO mouse (disrupted estrogen receptor α), the β ERKO mouse (disrupted estrogen receptor β), as well as the α/β ERKO mouse (disrupted estrogen receptors α and β ; ref. 73). These model systems are currently used by several research groups and allow scientists to study the role of aromatase and the individual estrogen receptors *in vivo*.

Plasma and Tissue Estrogen Levels

Plasma estrogens of interest are E_2 , estrone (E_1), and estrone sulfate (E_1S). Whereas only E_2 is biologically active, plasma levels of E_1 (the main product of aromatization in postmenopausal women) and E_1S are about 4- and 20-fold higher than the concentration of E_2 , respectively, in postmenopausal women (74, 75). As a consequence, E_1S is a useful surrogate variable due to a ratio of >100 between the plasma levels of E_1S in postmenopausal women and the sensitivity limit for E_1S analysis (29).

The importance of plasma estrogens as an intratumor source as well as the validity of using plasma estrogen suppression as a variable for aromatase inhibitor efficacy remains controversial. There are several reasons for this. First, the mean tissue level of E_2 is an order of magnitude higher than plasma level in postmenopausal women, with particular high levels in many breast tumors (76, 77). Because plasma levels of E_1S are 10- to 20-fold higher than plasma E_2 (78), circulating E_1S has been suggested a major estrogen source to the tumor tissue (79). This

hypothesis is partly challenged by contemporary findings that breast intratumor levels of E_1S are less than half the level of E_2 (77). On the other hand, the identification of an active transport mechanism for E_1S across the cell membrane (80) and high expression of sulfatase (81) as well as 17β -dehydrogenase type 1 (82, 83) in tumor tissue may be consistent with a rapid uptake of E_1S followed by conversion to E_2 . Whereas one study suggested an active uptake of E_2 from plasma into tumor tissue in rats (84) and studies evaluating the contribution of circulating estrogens to intratumor levels in breast cancer patients have reported a substantial interindividual variation (85, 86), the general opinion is that the high concentration of E_2 in tumor tissue is due to high expression of the aromatase enzyme leading to local estrogen synthesis from androgen precursors (Fig. 3; ref. 56).

The finding of a high tissue to plasma gradient, in particular for E_2 , has questioned the validity of measuring plasma estrogen levels as well as total body aromatization as surrogate markers for tissue estrogen levels. Postmenopausal estrogen synthesis occurs in most nonglandular tissues including fat, muscle, liver, and skin, and in normal breast as well as malignant tissue (87–101). All circulating estrogens in postmenopausal women are synthesized in nonglandular tissue compartments, whereafter they leak into the circulation by a passive gradient for subsequent removal by the liver and kidneys (102). Whereas interindividual variation in estrogen clearance rates (103) would influence plasma hormone levels, the drop in plasma levels recorded in relation to drug therapy reflects an effect on total body estrogen production and, thereby, tissue production in general. Whereas the issue has sometimes been a subject to discussion, circulating estrogens thus reflect tissue synthesis and may be considered as surrogate markers for normal tissue levels.

Intratumor estrogen levels remain a different topic. Aromatase expression may be particularly high in tumors (86), and the observation made by different groups, including our own (77), of a lack of correlation between plasma and intratumor estrogen levels may reflect a substantial interindividual variation with respect to local (tumor) estrogen synthesis.

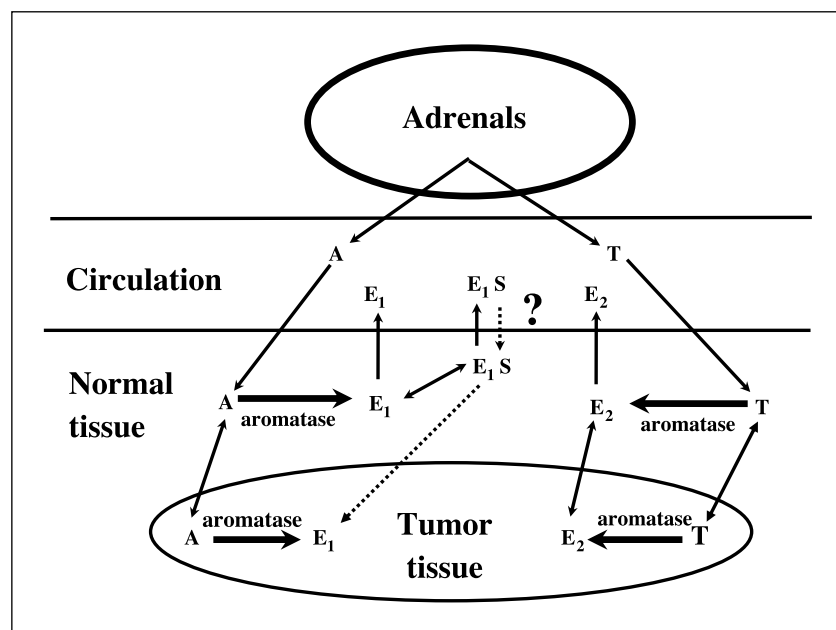
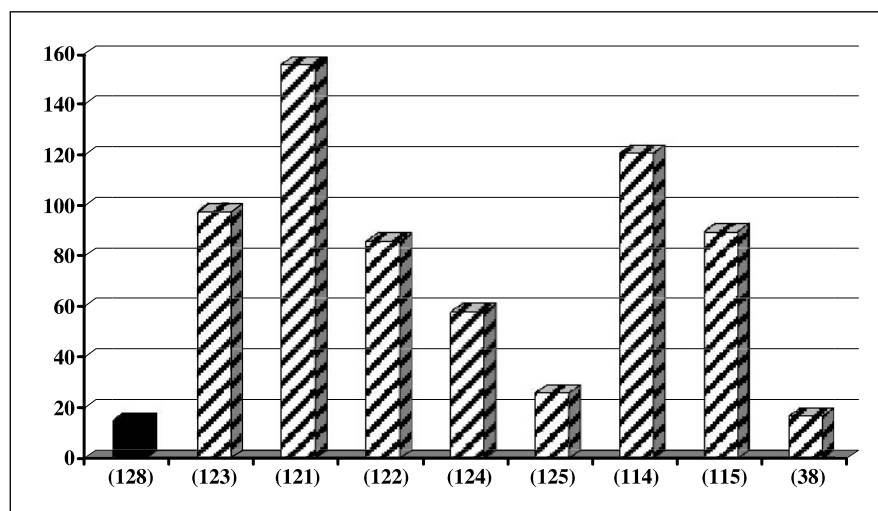


Fig. 3. Origin of breast cancer tissue estrogens and sites of action of aromatase inhibitors and inactivators.

Fig. 4. Plasma E_2 levels (pmol/L) in postmenopausal breast cancer patients as published in the literature (in chronological order). The first (black) column presents the theoretical plasma E_2 value calculated from androgen production rates, plasma hormone levels, and mean level of aromatization [see Lønning et al. (128) for details].



Whereas the aromatase enzyme is encoded by a single copy of the *CYP19* gene localized at human chromosome 15q21.2 (104), a total of nine promoters have been identified thus far; these are differentially expressed in different tissues (105, 106). The promoters I.3, II, and I.7, which dominate in breast cancer tissue (107), may be stimulated by interleukins (interleukins 6 and 11) as well as tumor necrosis factor α and prostaglandin 2 (108, 109), all commonly expressed in tumor tissue. Whereas a limited number of studies have evaluated intratumor estrogen suppression during treatment with aromatase inhibitors (110), data thus far suggest a similar degree of estrogen suppression in tumors expressing high hormone levels and in plasma (77), arguing against a local escape phenomenon.

Determination of plasma and tissue estrogen levels. Due to successful development of sensitive assays for plasma estrogens, such methods in general have replaced traditional methods of urinary estrogen measurements (111). Notably, there is no international consensus regarding normal values for plasma estrogen levels in postmenopausal women. Whereas studies by different groups have reported high plasma estrogen levels to be a significant risk factor for breast cancer development in postmenopausal women (112–120), the fact that the mean values of E_2 recorded by different investigators may vary by a factor of up to 10 (refs. 38, 114, 115, 121–125; Fig. 4) questions the validity of doing a meta-analysis on these data (126, 127). Theoretical calculations based on androgen production, androgen and estrogen plasma levels, aromatase activity, and hormone clearance rates suggest plasma levels of E_2 to be in the range of 10 to 20 pmol/L (128), consistent with values reported by groups using highly sensitive RIAs for estrogen measurements (38).

Considering RIA methods in general, the major problems are lack of sensitivity in general and lack of specificity against potential cross-reactive compounds in particular; each factor may result in false high values. Using highly sensitive RIAs, we and others recorded mean values for E_2 in the range of 15 to 25 pmol/L, with mean levels of E_1 and E_1S of about 100 and 4 to 500 pmol/L, respectively (50, 77). However, taking into account sensitivity limits in the range of 2 to 3 pmol/L (somewhat higher for E_1), this means that for the majority of patients we are able to record plasma hormone suppression below 1% of pretreatment values with respect to E_1S , but not for E_2 or E_1 (29). Thus, the

measurement of E_1S in plasma during treatment with aromatase inhibitors is a useful surrogate variable for plasma estrogen suppression in general. This is illustrated by a recent study comparing the two third-generation aromatase inhibitors anastrozole and letrozole in a crossover design (38). Here, we found a significant difference with respect to plasma levels of E_1 and E_1S during treatment with the two compounds but no difference in plasma levels of E_2 (Fig. 5). This was due to the fact that 9 of 12 and 12 of 12 patients had their plasma E_2 levels suppressed below the sensitivity of the method during treatment with anastrozole and letrozole, respectively. We did not evaluate the clinical responses comparing anastrozole and letrozole due to the study design with small numbers of patients and short-term exposure to the compounds. However, a direct comparison of the clinical effects of anastrozole versus letrozole has been recently published by Rose et al. (129).

Due to their steroidal structure, aromatase inactivators and their metabolites are particularly prone to cross-reactivity in the RIAs. Whereas interactions involving the mother compound and major metabolites can be tested for, it is difficult to exclude potential minor, probably unidentified, cross-reacting metabolites. The daily dose of exemestane for clinical treatment is 25 mg, and total estrogen synthesis in postmenopausal women about 50 μ g daily (111). Assuming aromatase inhibition of about 98% (130), a metabolite of exemestane accounting for 1% of its metabolism with a plasma clearance rate resembling E_2 and a cross-reactivity against the antibodies of 10% may in theory increase the value of plasma E_2 measured during therapy by 100% (128). The problem of metabolite interaction is illustrated by the fact that plasma estrogen levels in patients treated with exemestane can be measured only after high-performance liquid chromatography purification of the plasma samples (57).

The problems related to sensitivity of the assays are of relevance to tissue estrogen measurements as well. However, due to different levels of the individual estrogens in plasma and tissue and the sensitivity of the individual assays, E_2 seems to be the most reliable indicator for assessment of alterations in tissue estrogen levels (29, 77, 131). Notably, tissue estrogen measurements are time and resource consuming (131), for which reason such studies may include a limited number of patients only.

Measurement of in vivo aromatization by use of isotope techniques. This may in principle be done by two methods:

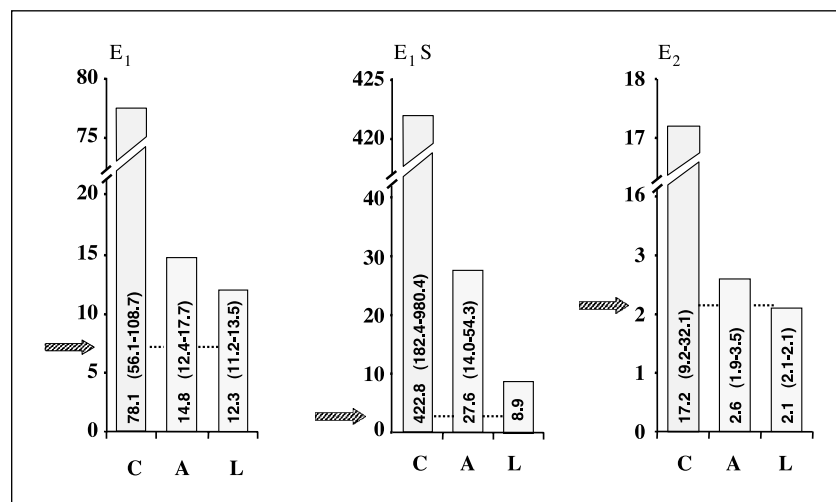


Fig. 5. Plasma E₁, E₁S, and E₂ levels (pmol/L) in postmenopausal breast cancer patients treated in a crossover trial [see Geisler et al. (38) for details] comparing anastrozole (A) and letrozole (L). C, baseline. Arrows indicate detection limits of the RIAs.

assessment of aromatase inhibition by measuring the isotope ratio in plasma E₁ during steady-state infusion (26) or by measuring the isotope ratio in the main estrogen metabolites extracted from the urine following an i.v. bolus injection.

In all studies done thus far, the tracers used have been ³H-labeled androstenedione and ¹⁴C-labeled E₁. With the plasma method, the aim is to achieve a steady-state level through concomitant infusion of the two tracers (26, 132, 133). With the urine method, a bolus injection of ³H-labeled androstenedione and ¹⁴C-labeled E₁ is followed by total urine collection over a period of 4 days with isolation and measurement of the isotope ratio in the major estrogen metabolites (34). For practical reasons, this may be limited to E₁ and estriol (E₃), leaving out the major catechol (2-hydroxylated) metabolites. The reason is that these metabolites need to be protected from oxidation and epoxide formation following glucurolysis (134).

Whereas different research groups have determined the *in vivo* aromatase inhibition capacity of different compounds (26, 47, 135, 136), these groups have used different methods, for which reason the results are not directly comparable.

In a collaborative program between the Royal Marsden Hospital and our group, we determined the *in vivo* aromatase inhibition with a panel of first-, second-, and third-generation inhibitors (37, 38, 130, 137-142). The results are summarized in Table 2. Notably, whereas first- and second-generation compounds in general caused aromatase inhibition <90%, the third-generation compounds caused a mean aromatase inhibition ≥98%. It is important to underline that the differences between first-, second-, and third-generation compounds concerning aromatase inhibition are not due to improvements in methodology but mirror a significant difference in efficacy between these compounds. The tracer method used in collaboration by our groups in Bergen and in London (head: Prof. M. Dowsett) has been used without any major changes from the time first published more than a decade ago (34). However, whereas a dose-response effect has been evaluated for several compounds (Table 2), a direct inpatient comparison of two different drugs has been done for anastrozole and letrozole only. Notably, the method was able to discriminate the biochemical efficacy of these highly potent compounds, providing indirect evidence for its sensitivity (38).

Is *In vivo* Assessment of Endocrine Effects of Clinical Importance?

This important question may be addressed in two different ways. (a) Are we able to differentiate potential differences between compounds and, if so, are those differences related to clinical efficacy? (b) Do we have evidence suggesting any correlation between the degree of estrogen suppression and clinical outcome among individual patients treated with the same compound?

Based on the tracer results reported above, aromatase inhibitors may be divided into contemporary third-generation compounds (anastrozole, exemestane, and letrozole), causing ≥98% inhibition, and the first- and second-generation compounds, causing 85% to 90% inhibition. Whereas the trials comparing second-generation compounds to progestins and tamoxifen included a limited number of patients by today's standards (143-147), there is no indication from these data suggesting improved clinical efficacy of any of these aromatase inhibitors compared with conventional treatment. In contrast, despite some diverging observations between individual trials, in general the superiority of the third-generation compounds has been established (see ref. 148 for a detailed review of these trials). Notably, whereas we found letrozole to enhance aromatase inhibition and plasma E₁ as well as E₁S suppression when compared with anastrozole in a crossover-designed study (Fig. 5; ref. 38), it is not clear whether this difference translates into an improved clinical efficacy (129).

Two articles have addressed the issue of correlation between degree of plasma estrogen suppression and clinical outcome (149, 150). The first study (149) was conducted more than 20 years ago during the pioneering period of development of aminoglutethimide; the second was reported more recently, and compared the degree of estrogen suppression to clinical outcome in patients treated with formestane (150). Notably, in that study (150) plasma estrogen levels were measured without doing high-performance liquid chromatography purification (57) of the samples before RIA, and the contrast between their finding (mean estrogen suppression of 40-60%) and the degree of aromatase inhibition (85-90%) recorded with tracer methods (151) suggests estrogen measurement in the study

done by Bajetta et al. (150) to be disturbed by cross-reacting metabolites.

At this stage, the issue whether there may be any correlation between degree of estrogen suppression and the likelihood of achieving a response among individuals treated with the same aromatase inhibitor remains unsettled. Taking into account the high number of potential mechanisms of resistance to endocrine therapy in general (172), such a study, apart from requiring suitable assays combining the requirement of analytic simplicity and high sensitivity, would need a substantial number of patients to address the issue properly. Even if a correlation could be found, it remains unlikely that such a test would be routinely implemented to select patients for therapy or to define patient drug dosing at an individual level.

However, another interesting question remains to be addressed. Realizing that premenopausal as well as postmenopausal women with breast cancer respond to estrogen suppression, and that the third-generation inhibitors are more effective compared with the less potent first- and second-generation ones, these observations indirectly suggest a dose-response relationship between degree of estrogen suppression and clinical efficacy. Notably, long-term estrogen deprivation of hormone-sensitive MCF-7 cells *in vitro* may sensitize these cells to E₂ concentrations 10⁻⁴ to 10⁻⁴⁻⁵ times the concentration normally required for maximal growth stimulation (152). Assuming third-generation aromatase inhibitors and inactivators to reduce estrogen production by 98% to 99%, we may ask the question whether a more potent inhibition (e.g., 99.9%) will cause a better clinical outcome. And if so, should such a therapy be implemented following relapse on a third-generation inhibitor, or given as first-line treatment? Would more aggravated estrogen suppression further sensitize the tumor cells to subsequent additive therapy with estrogens in pharmacologic doses (see later)? Whereas these questions are of clinical as well as biological relevance, our main problem is the technical incapacity to assess the endocrine effects of aggravated hormone suppression *in vivo* with the methods available today.

Lack of cross-resistance between aromatase inhibitors and inactivators. An interesting clinical observation is the rather surprising finding of a lack of complete cross-resistance be-

tween nonsteroidal aromatase inhibitors and the steroidal aromatase inactivators. The issue has been reviewed in detail elsewhere (153); only the key points will be summarized here. Whereas the response rate in general is low, there is no doubt that a significant number of patients may achieve durable stable disease if treated with a steroidal inactivator following a nonsteroidal compound; this effect is not explained through a more extensive suppression of plasma estrogens or total body aromatization. The most obvious reason for non-cross-resistance between steroidal and nonsteroidal drugs is the major difference in their biochemical structure. Another mechanism could be the slight androgenic effects caused by the steroidal compounds or some of their metabolites, illustrated by the dose-dependent drop in sex hormone binding globulin seen with exemestane due to its 17-hydroxymetabolite (57, 154). Thus, an important aim for future studies is to explore the mechanisms behind this interesting observation.

From what has been discussed above, it seems unlikely that we should be able to detect differences in plasma or tissue estrogen levels between groups of patients treated with third-generation aromatase inhibitors and inactivators. However, any difference with respect to clinical effects should be reflected in different biological effects at the tumor level. Thus, an issue for future studies could be to assess alterations in gene transcription in relation to treatment with compounds of the different classes using techniques like cDNA microarrays (155, 156).

Estrogen suppression and selective estrogen receptor modulators: combination versus sequential therapy. Whereas attempts to combine different types of endocrine therapy have in general been unsuccessful (157-163), lessons from some recent clinical studies provide important information to our understanding of the mechanisms by which estrogen suppression provides antitumor effects *in vivo*. The combination of tamoxifen and a luteinizing hormone-releasing hormone analogue was found superior to tamoxifen monotherapy in treatment of premenopausal patients suffering from metastatic breast cancer (30, 164). Whereas the issue of mechanisms of action and resistance to selective estrogen receptor modulators is beyond the scope of this article, certain biological aspects are of interest to our understanding of the antitumor effects of estrogen deprivation and will therefore be discussed here.

Table 2. Effects of different aromatase inhibitors and inactivators on whole-body aromatization

Compound	Drug dose in mg	% Aromatase inhibition	Ref.
Aminoglutethimide	250 qid	90.6%	(138)
Formestane (p.o.)	125 od/125 bid/250 od	72.3%/70%/57.3%	(140)
Formestane (i.m.)	250 2w/500 2w/500w	84.8%/91.9%/92.5%	(137)
Rogletimide	200 bid/400 bid/800 bid	50.6%/63.5%/73.8%	(138)
Fadrozole	1 bid/2 bid	82.4%/92.6%	(37)
Anastrozole	1 od/10 od	96.7%/98.1%	(142)
	1 od	97.3%	(38)
Letrozole	0.5 od/2.5 od	98.4%/98.9%	(141)
	2.5 od	>99.1%	(38)
Exemestane	25 od	97.9%	(130)

NOTE: All values are determined by the same assay at the Academic Department of Biochemistry, Royal Marsden Hospital, London, United Kingdom (Head: Prof. M. Dowsett) and the Breast Cancer Research Group at the Haukeland University Hospital in Bergen, Norway (Head: Prof. P.E. Lønning). Abbreviations: od, once daily; bid, twice daily; qid, four times daily; w, weekly; 2w, every 2 weeks.

Whereas tamoxifen is an active compound in premenopausal women (165), due to disturbance of ovarian function, it increases plasma E_2 levels by a factor of 2 to 4 (166–169). Thus, it has been questioned whether this effect could be detrimental to the clinical outcome, consistent with the finding that addition of a luteinizing hormone–releasing hormone analogue improved tamoxifen efficacy. In contrast, tamoxifen does not influence plasma levels of E_2 in postmenopausal women (170). In the recent Arimidex, Tamoxifen, Alone or in Combination study, anastrozole was found superior to tamoxifen monotherapy preventing relapses (31). Here, tamoxifen and anastrozole in combination did not improve outcome compared with tamoxifen monotherapy, suggesting a detrimental effect of tamoxifen in postmenopausal patients treated with anastrozole. Taken together, these results are consistent with the hypothesis that plasma E_2 in premenopausal concentrations may be detrimental by competing with tamoxifen for the estrogen receptor. Contrary, in postmenopausal women experiencing supralow estrogen levels during treatment with an aromatase inhibitor, the estrogen agonistic effect of tamoxifen (171) may be detrimental. Thus, combining these data provides important information to our understanding of the “antiestrogenic” effects of selective estrogen receptor modulators *in vivo*.

Perspectives on the Future; Understanding the Mechanisms of Resistance to Estrogen Deprivation

A major obstacle to the clinical use of endocrine therapy is the occurrence of primary or acquired endocrine resistance. Whereas the issue in general has been discussed in several recent publications (172–178) and would be beyond the scope of this article, we will briefly summarize certain topics of relevance to resistance to estrogen deprivation in general and treatment with aromatase inhibitors in particular.

Contemporary clinical research is focusing on optimizing adjuvant treatment with aromatase inhibitors (the design of the major studies is summarized in Fig. 6), taking into consideration important issues like potential differences regarding compound efficacy, drug administration (sequential treatment versus monotherapy), duration of therapy, and side effects. Although several trials involving aromatase inhibitors in the adjuvant setting are still ongoing, three major adjuvant studies involving anastrozole, letrozole, and exemestane have been published recently (31, 39, 40). In conclusion, all three trials reported superiority of aromatase inhibitors either given alone (Arimidex, Tamoxifen, Alone or in Combination) or in sequence with tamoxifen (OEXE-31 and MA-17) compared with tamoxifen alone.

Thus, future research exploring the mechanisms of action and resistance to aromatase inhibitors may potentially lead to even more effective strategies. Here, the potential differences between steroidal aromatase inactivators (like exemestane) and the nonsteroidal aromatase inhibitors, manifested by the lack of total cross-resistance between compounds of the two classes (179), merit special consideration. The fact that exemestane, in addition to being a potent aromatase inactivator, expresses weak androgen-agonistic effects through its main metabolite *in vivo* (57) indicates that exemestane may provide additional mechanisms acting in concert with

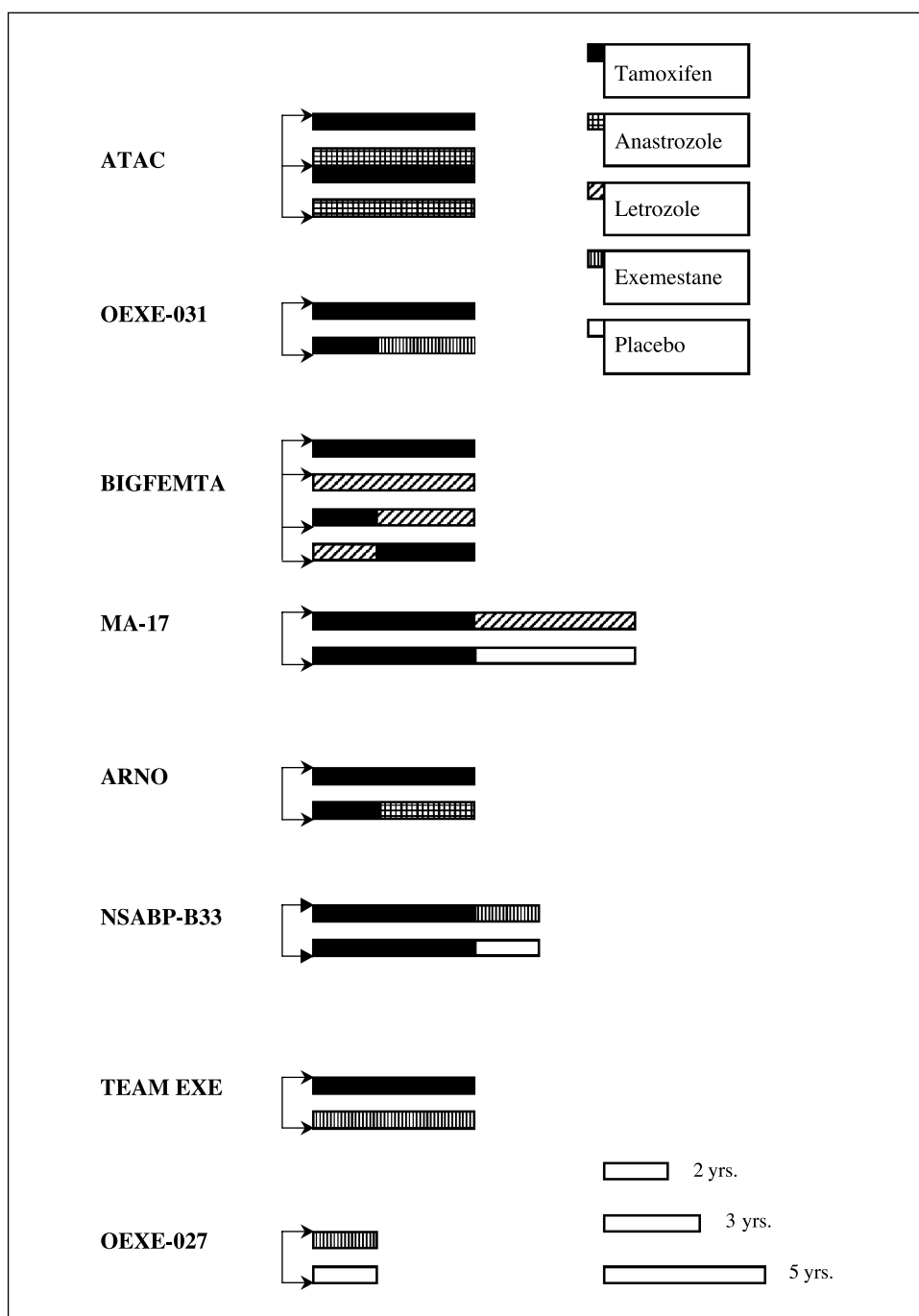
estrogen deprivation (180). In addition, recent findings by Dr. Brodie’s group, suggesting combined treatment with exemestane and tamoxifen to be superior to either tamoxifen or exemestane given as monotherapy in a mouse tumor model (181), underline the importance of exploring the consequences of structural differences between aromatase inhibitors and inactivators.

The extension of basic biological knowledge, together with methodologic improvements, offers the possibility to explore the mechanisms of therapy resistance *in vivo* in a manner we could just dream about 10 to 20 years ago (155, 182, 183). Importantly, whereas experimental data may generate hypotheses considering the mechanisms of therapy resistance, the proof is to verify the concepts *in vivo*—by translational research. Here, we will focus on selected issues including the mechanisms of estrogen sensitization, cross-talks between hormonal regulation and other growth factor pathways, and the potential of aggravated aromatase inhibition.

Several authors have shown that MCF-7 cells grown in culture may adapt to a low estrogen environment by developing hormonal hypersensitivity (152, 184, 185), achieving maximal growth stimulation by an E_2 concentration 10^{-4} to 10^{-5} times the concentration needed for the mother cell line. Whereas the mechanism is not fully understood, several signaling pathways, including mitogen-activated protein kinase, seem to be involved (186). Estrogen stimulation *in vitro* is following a bell-shaped curve inasmuch as estrogens in concentrations above the level required for maximal growth stimulation inhibit cellular growth. This bell-shaped profile was also recorded for the hypersensitized cells, inasmuch as these cells could be growth inhibited by adding E_2 in concentrations that would have stimulated the growth of the wild-type cells. The concept is substantiated through clinical observations: before the era of contemporary endocrine therapy, estrogens in high doses were used for breast cancer therapy (187–189). Based on the hypothesis that estrogen deprivation could sensitize tumors to treatment with estrogens in pharmacologic doses, we exposed patients whose tumors had developed resistance to aromatase inhibitors to estrogens in high doses (diethylstilbestrol, 5 mg thrice daily). During this regimen, we recorded an objective response in 10 of 29 patients, some of them lasting for >2 years (190). Our challenge is to explore molecular changes in tumors exposed to such therapy elucidating the mechanism of action. Exact knowledge about these mechanisms, based on translational research, may open for new therapeutic strategies.

There is a growing body of literature suggesting that cross-talk between growth factors and estrogen receptors may contribute to endocrine resistance (176, 186, 191, 192). Of particular interest are some recent findings with respect to human epidermal growth factor receptor 2 overexpression, suggesting this may reduce responsiveness to tamoxifen (193) but not to aromatase inhibition (194, 195). Of particular interest is an article by Zhu et al. (195), revealing treatment with aromatase inhibitors to eradicate human epidermal growth factor receptor 2–positive cells *in vivo*. In addition, recent findings by Modlich et al. (196) suggest that the time points for any gene expression analyses are very critical as immediate changes in gene expression might occur after the initiation of therapy. All in all, a better understanding of the multidirectional cross-talk between

Fig. 6. Adjuvant trials evaluating the effects of aromatase inhibitors or inactivators in early breast cancer.



growth factor pathways, the estrogen receptors (both at the genomic and nongenomic level), and estrogen receptor cofactors is currently one of the most promising scientific attempts to overcome endocrine resistance (191).

A final issue relates to aggravated hormone suppression. Prostaglandin E₂, the product of cyclooxygenase 2 (COX-2), has been shown to be one of the most important factors controlling aromatase expression *in vitro* via promoter II (109), and aromatase expression is strongly correlated with COX-2 expression in series of breast cancer patients (61). Whereas COX-2-derived signals influence cell cycle progression, invasiveness, and angiogenesis in preclinical trials (197), the potential influence on estrogen synthesis provided the main background

for implementing celecoxib into adjuvant and breast cancer prevention trials in concert with aromatase inhibitors. This approach, however, has several pitfalls. Whereas COX-2 overexpression has been associated with a significant inferior disease-free survival in subsets of breast cancer patients (see ref. 197 for references), any association between expression of a certain biological variable and prognosis could be misleading regarding biological interpretation. Often, a prognostic factor may be just a covariate to other biological variables (182), illustrated by the finding that expression of the estrogen receptor—the target for one of the most potent mitogens in breast cancer—has been associated with an improved prognosis also in breast cancer patients not exposed to adjuvant

endocrine therapy (198, 199). Thus far, there has been no study reporting intratumor estrogen levels in patients treated with a COX-2 inhibitor. Even if the concept—combining a COX-2 inhibitor with an aromatase inhibitor—may improve therapeutic outcome, there is a need to explore the mechanisms determining intratumor hormone disposition (as well as the effects on other potential targets).

Conclusion

As shown in this review, translational research has played a key role during the successful development of aromatase inhibitors for breast cancer treatment. Following the implementation of aromatase inhibitors and inactivators of the third-generation as a first-line therapy in metastatic breast cancer, recent

findings of large studies evolving the role for aromatase inhibitors in the adjuvant setting have caused optimism. However, whereas the adjuvant studies provided further evidence for the superiority of aromatase inhibitors compared with tamoxifen, the clinical improvements are in fact marginal, although statistically significant. Moreover, these studies are not designed to elucidate fundamental biological mechanisms leading to the major problem in the clinical use of these drugs: endocrine resistance. However, with the merging knowledge from basic research, together with method developments in molecular biology, we are now facing the opportunity of exploring the mechanisms of resistance to estrogen deprivation. Again, carefully designed translational research protocols are the most promising tools of physicians and basic scientists to change the situation for breast cancer patients fundamentally.

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