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Lack of Inhibition of Placental Estrone Sulfatase and Aromatase Enzymes by Vitamin D_3 and Its Analogs

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The aromatase and estrone sulfatase enzymes are important sources of biologically active estrogens in postmenopausal women with breast cancer. Promising initial results in the treatment of endocrine-responsive breast cancer have been exhibited by 1α25-dihydroxyvitamin D_3 and the synthetic vitamin D analogues MC903 and EB1089. However, these compounds together with vitamin D_3 and vitamin D_3 sulfate did not inhibit the human placental aromatase enzyme when assayed up to 20 μm. Only vitamin D_3 sulfate and 1α25-dihydroxyvitamin D inhibited the estrone sulfatase activity in human placental microsomes, albeit at high concentration (32 and 37% inhibition, respectively with 50 μm each inhibitor). It is unlikely that inhibition of aromatase or estrone sulfatase enzymes contribute to the inhibitory effect of this group of compounds on breast cancer cells *in vivo*.

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INTRODUCTION

Approximately one third of human breast carcinomas are hormone-dependent [1], and estrogens are the most important hormones involved in supporting growth of hormone-dependent breast tumors [2,3]. Plasma levels of estrone and estradiol in postmenopausal women are very low. However, the estrogen concentration in breast tumor tissues is of an order of magnitude higher than in the plasma [4], suggesting local intratumoral production of estrogen in breast cancer cells from precursor substrates. The predominant source of estrogen production in postmenopausal women is the extraglandular conversion of androstenedione to estrone by the aromatase enzyme in peripheral tissues [5], and intratumoral aromatase activity has been demonstrated in breast carcinoma samples [6–9]. Moreover, aromatase inhibition is a useful therapeutic option in advanced breast cancer [10].

However, estrone sulfate is the most abundant estrogen in peripheral blood [11], and can be taken up by breast cancer MCF-7 cells and hydrolyzed by estrone sulfatase in sufficient amounts to stimulate a biological response [12]. Intratumoral estrone sulfatase activity is also present in breast carcinoma tissue [13–16], and a comparative study of estrone sulfatase and aromatase activities in human breast cancer samples suggested that the estrone sulfatase pathway is possibly a more abundant source of intratumoral estrogens [17].

In recent years it has been suggested that the active form of vitamin D_3, namely 1α25-dihydroxyvitamin D_3 exerts effects on a variety of tissues apparently unrelated to calcium homeostasis [18], including effects on cellular oncogene transcription and growth factor receptor expression *in vitro* [19, 20]. It also inhibits the proliferation of estrogen receptor-positive MCF-7 and T47D breast cancer cells [21,22]. The synthetic analog 1αhydroxyvitamin D_3 which is converted to 1α25-dihydroxyvitamin D_3 *in vivo*, inhibits the progression of carcinogen-induced rat mammary tumors [23], and 25% of patients with locally advanced or cutaneous metastatic disease treated topically with MC903, a synthetic vitamin D analog [24] showed a response of the treated lesions [25]. A further analog, EB1089, which like MC903, is characterized by a modification of the C17 side chain of the vitamin D_3 molecule, has shown potent inhibition of MCF-7

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breast cancer cells *in vitro*, and regression of NMU-induced rat mammary tumors *in vivo* [26]. Vitamin D_3 sulfate is not hydrolyzed by estrone sulfatase, but has been reported to strongly inhibit its activity [27]. Consequently we considered it pertinent to further investigate vitamin D_3, vitamin D_3 sulfate and the analogs 1α25-dihydroxyvitamin D_3, MC903 and EB1089, as putative inhibitors of estrone sulfatase, and whether inhibition of the aromatase or sulfatase enzymes is contributory to the anti-tumor effect of this group of compounds in breast cancer.

EXPERIMENTAL

Reagents

[1β-^{3}H]Androstenedione (sp. act. 28 Ci/mmol) was obtained from New England Nuclear and radiochemical purity determined by thin layer chromatography in dichloromethane–diethyl ether (9:1). NADP (tetrasodium salt), glucose-6-phosphate, and glucose-6-phosphate dehydrogenase were purchased from Boehringer Mannheim, (Lewes, England). [6,7-^{3}H]-Estrone sulfate (sp. act. 47.7 Ci/mmol) was purchased from New England Nuclear (Du Pont, England). Purity was checked by thin layer chromatography (silica gel Merck S415 Kieselgel F254) using the following solvent system; ethyl acetate–methanol–ammonium hydroxide (75:25:2). [4-^{14}C]Estrone (sp. act. 60 Ci/mmol) was purchased from Amersham International (Amersham, England). Unlabeled estrone sulfate, unlabeled androstenedione, 1α25-dihydroxyvitamin D_3, 1α25-dihydroxyvitamin D_3 sulfate, and activated charcoal were purchased from Sigma Chemical Co. (Poole, England). EB 1089 and MC903 were kindly donated by Leo Pharmaceutical Products (Ballerup, Denmark). Precoated silica gel thin layer chromatography plates, solvents and other chemicals were purchased from BDH chemicals (Poole, England).

Tissue and tissue preparations

A full term human placenta was obtained immediately after delivery from the labor ward of St George's Hospital, London and transferred to the laboratory on ice for preparation of tissue fractions. Placental microsomes were prepared for aromatase [28] and sulfatase [29] assays as described previously. Estrone sulfatase and aromatase assays were performed as described previously [29, 30]. Briefly, estrone sulfatase activity was assayed by measuring the ^{3}H-labeled non-polar metabolites formed from [^{3}H]estrone sulfate by solvent partition [29]. A stock solution of each compound dissolved in DMSO was prepared and added to the assay tubes such that the solvent did not succeed 2% of the total volume. An equal volume of DMSO was added to the control tubes. Each compound was assayed over a range of concentrations up to 50 μm of added inhibitor, using a saturating substrate concentration of 20 μm. The estrone sulfatase activity was determined from the linear plots of product released against time, comparison of this value with the control activity enabled the percentage inhibition to be determined.

Aromatase activity was determined by quantifying the tritiated water released from [1β-^{3}H]androstenedione during aromatization to estrone. Each assay tube (1 ml) contained 50 mm potassium phosphate buffer pH 7.4, co-factors and the substrate solution in propanediol (2.5% UV). Unlabeled androstenedione was combined with the labeled steroid (0.5 μCi) to give a final substrate concentration of 0.38 μm (K_m = 0–0.038 μm). The co-factor solution consisted of 1 mM NADP, 10 mM glucose-6-phosphate and glucose-6-phosphate dehydrogenase (2 U/ml). The reaction was started by the addition of placental microsomes. Aliquots (0.2 U) were removed after 5, 10 and 15 min of incubation and added to activated charcoal (1% w/v), 0.1% Tween 80, 0.2% dextran T70 and 1 mM mercuric chloride on ice. Tritiated water was separated from the unreacted steroids by centrifugation. All assays were run in duplicate and the compounds to be tested were dissolved in DMSO and added to the assay tubes to give 1% of the total volume.

For both assay systems, the IC_{50} value is the concentration of inhibitor required to reduce the activity of the enzyme to 50% of the control value.

RESULTS AND DISCUSSION

Enzyme characteristics and assay methods were validated as described previously [29, 30]. None of the compounds tested had any inhibitory effect on the placental aromatase assay over a range of concentrations up to 20 μm, so all IC_{50} values were greater than 20 μm. Similarly, EB1089, MC903 and vitamin D displayed no inhibitory effect on estrone sulfatase activity at final concentrations of 5, 10, 20 and 50 μm, that is, the IC_{50} is > 50 μm.

However, both vitamin D_3 sulfate and 1α25-dihydroxyvitamin D_3 resulted in an initial increase in estrone sulfatase activity at the lower concentrations, with a 42% increase in enzyme activity in the presence of 5 μm vitamin D_3 sulfate, and up to 70% increase in the presence of 10 μm 1α25-dihydroxyvitamin D_3. Thereafter, increased concentrations of both inhibitors resulted in inhibition of estrone sulfatase activity, with 32 and 37% inhibition at 50 μm of added vitamin D_3 sulfate and 1α25-dihydroxyvitamin D_3, respectively (Table 1), that is the IC_{50} is >50 μm for both these compounds. Previously, we have demonstrated that 5-androstene 3β,17β diol-3-sulfate is a potent inhibitor of estrone sulfatase [29], and this is confirmed in this current study, with a progressively greater inhibition with increasing concentration, with 80% inhibition of estrone sulfatase at 50 μm of added inhibitor (Table 1) and an IC_{50} of approx. 15 μm.

Previously we have shown from structure–activity studies that a sulfate group at the 3 position of the steroid structure is the most important feature of putative estrone sulfatase inhibitors [29], and it is not

Table 1. Inhibition of human placental estrone sulfatase by vitamin D_3 and analogs

Compound	% of control activity			
Conc:	5 μm	10 μm	20 μm	50 μm
EB 1089	100	100	100	100
MC 903	100	100	100	100
$1\alpha 25(OH)_2D_3$	168	176	83	63
Vitamin D_3	100	100	100	100
Vitamin D_3 sulfate	142	88	80	68
5-Androstene 3β,17β-diol-3-sulfate	96	60	30	20

Substrate concentration = 20 μm [6,7-^{3}H]estrone sulfate. All values are the mean of duplicate determinations of each of two time points.

surprising therefore that EB1089, MC903 and vitamin D are ineffective as estrone sulfatase inhibitors as they do not possess a sulfate group at this position. Similarly, although analogs of the natural substrate 4-androstene-3β,17β-dione are potent inhibitors of the aromatase enzyme [31], none of the compounds assayed have structures similar to this substrate, and are ineffective as inhibitors of aromatase. Both 1α25-dihydroxyvitamin D_3 and vitamin D_3 sulfate inhibit the estrone sulfatase enzyme, but only at high concentrations (50 μm), and even then the inhibition is less than that observed with some other 3-sulfated steroids, such as 5-androstene 3β,17β diol-3-sulfate. Consequently, these compounds are insufficiently potent for further development as inhibitors of estrone sulfatase. Moreover, at lower concentrations both these compounds stimulate estrone sulfatase activity by mechanisms which are unclear. It may be that these compounds, as a result of their chemical structure, possess detergent-like properties. It has been suggested that the human enzyme exists as a polymer composed of eight identical subunits within the microsomal membrane [32]. Previous studies have shown considerable differences in the physicochemical properties and inhibition kinetics of the human enzyme in the presence of Triton X-100 as compared with in detergent-free media, suggesting that detergents change the three-dimensional structures of the substrate-binding or catalytic sites of the enzymes [33]. Therefore the initial increase in enzyme activity may be due to the detergent nature of these compounds opening up the active site of the membrane-bound estrone sulfatase enzyme.

Inhibition studies of the estrone sulfatase enzyme in human placenta and human breast carcinoma tissue are sufficiently comparable so that the placental enzyme can be used to screen potential inhibitors [29]. Similarly putative inhibitors of human aromatase can be investigated using the placental enzyme [31], although expression of the aromatase gene may be regulated by different tissue-specific promoters in these tissues [34]. It is unlikely that inhibition of aromatase and estrone sulfatase enzymes contributes to the inhibition of growth of breast cancer cells observed by vitamin D_3 and its analogs *in vivo*.

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