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Vitamin D is a membrane antioxidant

Ability to inhibit iron-dependent lipid peroxidation in liposomes compared to cholesterol, ergosterol and tamoxifen and relevance to anticancer action

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Vitamin D is a membrane antioxidant: thus Vitamin D_3 (cholecalciferol) and its active metabolite 1,25-dihydroxycholecalciferol and also Vitamin D_2 (ergocalciferol) and 7-dehydrocholesterol (pro-Vitamin D_3) all inhibited iron-dependent liposomal lipid peroxidation. Cholecalciferol, 1,25-dihydroxycholecalciferol and ergocalciferol were all of similar effectiveness as inhibitors of lipid peroxidation but were less effective than 7-dehydrocholesterol; this was a better inhibitor of lipid peroxidation than cholesterol, though not ergosterol. The structural basis for the antioxidant ability of these Vitamin D compounds is considered in terms of their molecular relationship to cholesterol and ergosterol. Furthermore, the antioxidant ability of Vitamin D is compared to that of the anticancer drug tamoxifen and its 4-hydroxy metabolite (structural mimics of cholesterol) and discussed in relation to the anticancer action of this vitamin.

Vitamin D; Liposomal lipid peroxidation; Anticancer action; Tamoxifen; Membrane antioxidant; Cholesterol

1. INTRODUCTION

Vitamin D, which includes Vitamin D_3 (cholecalciferol) and Vitamin D_2 (ergocalciferol), is a lipid soluble vitamin, which is metabolized to the hormonally active $1,25(OH)_2$ derivative of cholecalciferol that has many biological effects including control of calcium and phosphorus metabolism [1]. There may, however, be other important biological effects of Vitamin D and although considerable effort has been put into studying the antioxidant action of Vitamin E (including α-tocopherol), which protects membranes [2,3] and LDL (low density lipoprotein) [4–6] against oxidative damage, the antioxidant potential of Vitamin D has not been investigated.

We have reported previously that the anticancer drug tamoxifen, which is widely used in the treatment, and recently in prevention, of breast cancer [7–11], inhibits lipid peroxidation in a range of systems [12–15] and protects human LDL against oxidative damage [16]. Tamoxifen apparently acts as a membrane antioxidant by structural mimicry of cholesterol (and ergosterol) thus enabling it to act in a similar way to stabilise the membrane against lipid peroxidation [13,14,17,18]. It is interesting, therefore, that Vitamin D, its active metabolite and also synthetic analogues have been reported to have anticancer activity [19–24]. Cholecalciferol, its metabolite 1,25-dihydroxycholecalciferol and 7-dehydrocholesterol are derived from and structurally related to cholesterol, whereas ergosterol is the parent compound of ergocalciferol and this led us to investigate their antioxidant abilities. The structures of these compounds are shown in Fig. 1.

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2. MATERIALS AND METHODS

2.1. *Preparation of liposomes*

Ox-brain phospholipid liposomes were prepared as described previously [13,25]. Liposomes were also prepared with and without the introduction of the compounds shown in Fig. 1., as described for cholesterol, ergosterol and tamoxifen [13,14,17,25]. Ox-brain phospholipid, Vitamin D_3 (cholecalciferol), Vitamin D_2 (ergocalciferol) and 7-dehydrocholesterol were purchased from Sigma. 1,25-Dihydroxycholecalciferol was a kind gift grom Dr. Lise Binderup, Leo Pharmaceutical Products Ltd., Ballerup, Denmark.

2.2. *Peroxidation of liposomes*

Reaction mixtures (final volume 1.0 ml) contained liposomes (0.1 ml), phosphate-buffered saline pH 7.4 (0.5 ml), water (0.2 ml) and 5 μl of ethanol or test compound dissolved in ethanol, except in the case of liposomes already containing introduced compounds. Peroxidation was started by adding freshly prepared aqueous solutions of $FeCl_3$ (0.1 ml) and ascorbate (0.1 ml) to give a final concentration of 100 μM of each. Incubations were carried out at 37°C for 20 min. At the end of the incubation 100 μl of a 0.2% (w/v) stock solution of BHT (butylated hydroxytoluene) in ethanol was added to suppress any further peroxidation during the TBA assay itself. The extent of lipid peroxidation was measured by the formation of thiobarbituric acid-reactive substances (TBARS) as described in [26]. HCl (0.5 ml of 25% v/v) was added to each reaction mixture, followed by 0.5 ml of thiobarbituric acid solution (1% w/v in 50 mM sodium hydroxide). After heating at

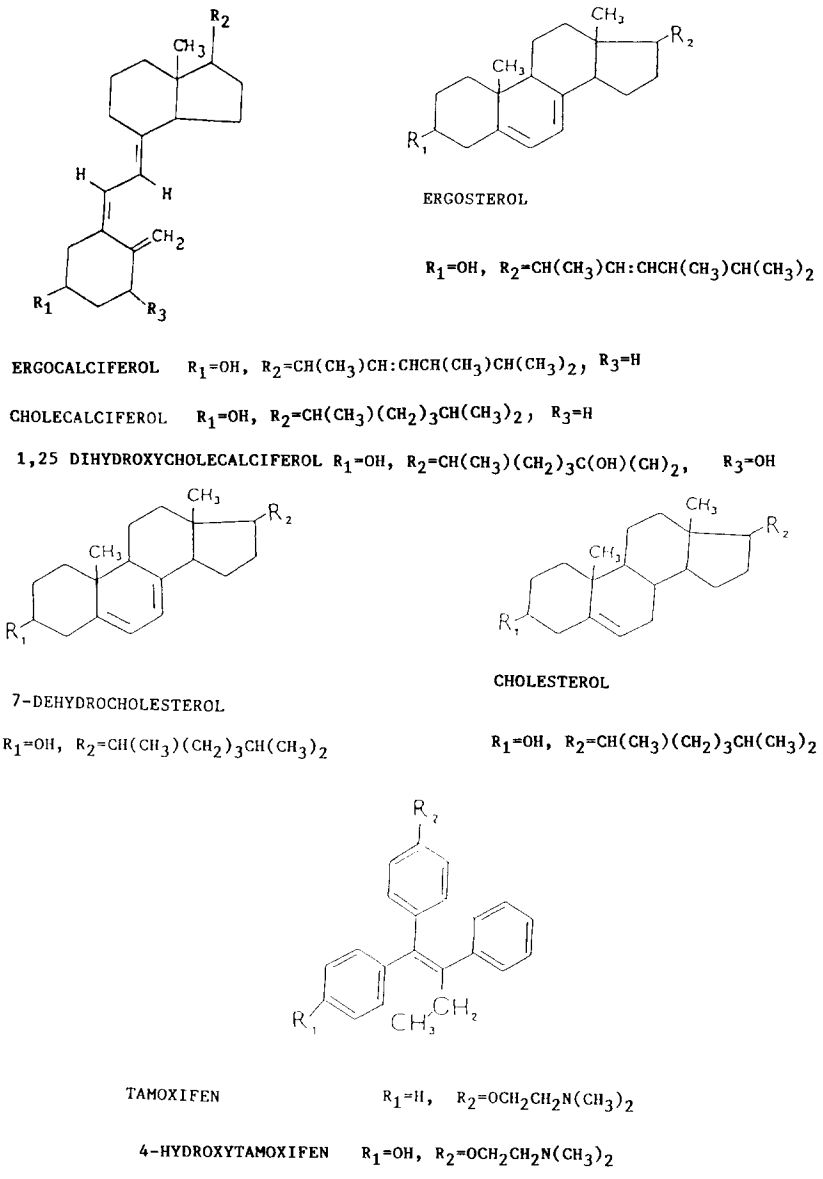

Fig. 1. Structures of Vitamin D compounds, tamoxifen and membrane sterols. Structural relationship of cholecalciferol, 1,25-dihydroxycholecalciferol and 7-dehydrocholesterol to cholesterol and of ergocalciferol to ergosterol.

80°C for 30 min the chromogen was extracted with 2 ml of butan-1-ol and the A_{532} of the upper (organic) layer was measured.

3. RESULTS

When added to preformed liposomes, ergocalciferol, cholecalciferol, 1,25-dihydroxycholecalciferol and 7-dehydrocholesterol each dissolved in ethanol had no effect on iron-dependent lipid peroxidation (see Table I), as found previously for cholesterol and ergosterol [13,14]. However, the introduction of cholesterol or ergosterol into ox-brain phospholipid liposomes during their preparation inhibited iron-dependent lipid peroxidation apparently by membrane stabilization [13,17,18] and we therefore studied the effect of similarly introducing cholecalciferol, 1,25-dihydroxycholecalciferol, ergocalciferol or 7-dehydrocholesterol. Fig. 2 shows that over the concentration range tested (up to 1 mM expressed as final concentration in the reaction mixture; compound:phospholipid ratio of 2.6×10^{-1}) ergocalciferol, cholecalciferol and 1,25-dihydroxycholecalciferol inhibited lipid peroxidation but were less effective inhibitors than 7-dehydrocholesterol. This inhibitory action was expressed as the IC_{50} values (see Table I) derived for the compounds tested and compared to those previously obtained for cholesterol, ergosterol, tamoxifen and 4-

Table I

IC_{50} values for the inhibition of liposomal lipid peroxidation by cholecalciferol, ergocalciferol, 7-dehydrocholesterol, tamoxifen, 4-hydroxytamoxifen, ergosterol and cholesterol

Compound/Drug	Liposomal systems	
	Final concentration in reaction mixture	
	(μM)	(mM)
	Added to preformed	Introduced into during preparation
Cholesterol	N.R.*	0.72*
7-Dehydrocholesterol	N.R.	0.34
Cholecalciferol	N.R.	1.0
1,25-Dihydroxycholecalciferol	N.R.	1.0
Ergosterol	N.R.*	0.054*
Ergocalciferol	N.R.	1.0
Tamoxifen	63*	0.050*
4-Hydroxytamoxifen	9*	0.0036*

N.R. = not reached, i.e. inhibition by the compound does not reach 50% and in the above examples the compounds do not in fact inhibit lipid peroxidation in the system indicated. *Data are quoted from reference [13]. Values are deduced from the graphs shown in Fig. 2 in which each point represents the mean ± S.D. of 3–5 separate assays.

hydroxytamoxifen [13,17,18]. Table I shows that the concentration of 7-dehydrocholesterol required to inhibit lipid peroxidation by 50% was approximately 2-fold less than for cholesterol. In contrast the IC_{50} values for cholecalciferol, 1,25-dihydroxycholecalciferol and ergocalciferol were 1.4 higher than for cholesterol. The IC_{50} values of ergosterol and tamoxifen were approximately 15-fold less than for cholesterol and that for 4-hydroxytamoxifen was 200-fold less. The IC_{50} value of ergocalciferol was 19-fold more than that of ergosterol.

4. DISCUSSION

Cholecalciferol (Vitamin D_3), its active metabolite 1,25-dihydroxycholecalciferol and ergocalciferol (Vitamin D_2) were found to be membrane antioxidants in this study, but were not as effective as cholesterol or ergosterol as inhibitors of iron-dependent lipid peroxidation when introduced into phospholipid liposomes (see Table I). 7-Dehydrocholesterol was twice as effective as cholesterol as an inhibitor of lipid peroxidation and its structure (see Fig. 1) shows it to have the ergosterol-type ring B: an extra double bond compared to cholesterol makes it more planar and thus potentially increasing interactions with phospholipid fatty acid side chains and indeed ergosterol is a more effective membrane antioxidant than cholesterol. However, 7-dehydrocholesterol is not as good a membrane antioxidant as ergosterol probably because it has the cholesterol-type side chain. In cholecalciferol (also 1,25-dihydroxycholecalciferol) and ergocalciferol, ring-B of the sterol nucleus has opened, thus reducing the potential for interaction with fatty acid side chains and resulting in decreased antioxidant ability compared to cholesterol and ergosterol, respectively. It is of interest that the two extra hydroxyl groups of 1,25-dihydroxycholecalciferol compared to cholecalciferol confer no extra antioxidant advantage on the metabolite compared to the parent compound. Cholecalciferol and ergocalciferol differ only in that they have the same side chains as cholesterol and ergosterol, respectively, but they are equally effective as inhibitors of lipid peroxidation and it can be postulated that while the more hydrophobic ergosterol-type side chain may aid incorporation into the liposomal bilayer in the optimal orientation for interaction of the sterol rings (particularly ring B) with the phospholipid side chains, it is the more planar ergosterol-type ring-B that is required for increased inhibition of lipid peroxidation through increased interaction with phospholipid fatty acid side chains.

Cholecalciferol, 1,25-dihydroxycholecalciferol, ergocalciferol and 7-dehydrocholesterol are likely to act as membrane antioxidants by stabilizing the membrane against lipid peroxidation via an interaction between their hydrophobic rings (although cholecalciferol, 1,25-

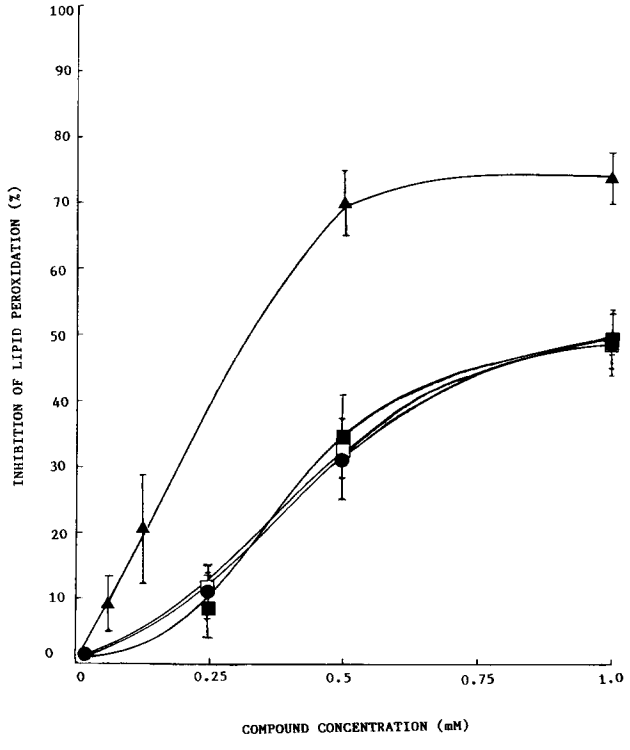

Fig. 2. Inhibition of lipid peroxidation by the compounds, (■) cholecalciferol, (□) 1,25-dihyroxycholecalciferol, (●) ergocalciferol and (▲) 7-dehydrocholesterol, each introduced separately into liposomes during their preparation. Results are mean ± S. D., n = 3–5.

dihydroxycholecalciferol and ergocalciferol have lost ring B) and the saturated, monounsaturated and polyunsaturated residues of the phospholipid fatty acid side chains, which decreases membrane fluidity as proposed previously for cholesterol, ergosterol and tamoxifen [13,14,17,18]. Indeed decreased membrane fluidity in cancer cells treated with tamoxifen has been reported [27].

Cholecalciferol, 1,25-dihydroxycholecalciferol, ergocalciferol and 7-dehydrocholesterol were all much less effective than the anticancer drug tamoxifen and its 4-hydroxy metabolite as inhibitors of lipid peroxidation and did not have any effect on peroxidation when added in ethanol to preformed liposomes instead of being introduced into the liposomes; a finding reported previously for cholesterol and ergosterol [13,14]. This is in contrast to tamoxifen and 4-hydroxytamoxifen, which are both potent inhibitors of lipid peroxidation under these conditions [13,14]. This difference may reflect the ease with which this anticancer drug and its 4-hydroxy metabolite are able to enter liposomes and natural membranes.

The $1,25(OH)_2$ metabolite (hormonal form) is recognised to be the active form of Vitamin D for calcium and phosphorus metabolism in the human body and we have shown in this study that it has an antioxidant ability, which may be of importance in protecting the membranes of normal cells against free radical-induced oxidative damage as it is possible that these highly lipophilic compounds may accumulate in membranes to achieve the concentrations found to inhibit lipid peroxidation in this study. Furthermore, by decreasing membrane fluidity by the membrane interaction that is thought to lead to the observed inhibition of lipid peroxidation, Vitamin D could help inhibit the growth of cancer cells (especially metastatic cells), which often have increased membrane fluidity compared to normal cells [28]. In this connection, Vitamin D is considered to be protective against colon cancer [19], Vitamin D metabolites have been reported to inhibit the growth of colon cancer cells in culture [21] and synthetic Vitamin D analogues appear to have antiproliferative effects on breast cancer cells in culture [23,24]. Furthermore, Vitamin D_3 is formed in humans by the action of sunlight on skin, and thus its antioxidant ability (and that of its precursor in skin, 7-dehydrocholesterol) should help protect the cells in which it is formed against the aging and carcinogenic effects of UV (ultra violet) light caused in part by peroxidation of cell membranes.

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