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Pharmacokinetic studies of vitamin D analogues: relationship to vitamin D binding protein (DBP)

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Vitamin D_3, 25-hydroxyvitamin D_3 ($25OHD_3$) and 1α,25-dihydroxyvitamin D_3 ($1\alpha,25(OH)_2D_3$) bind to the vitamin D binding protein (DBP) in the serum. During the development of synthetic vitamin D analogues, it has been shown that the majority of analogues bind to DBP with a low affinity. This modifies their biological activities *in vitro* compared to $1\alpha,25(OH)_2D_3$, since binding to DBP decreases the cellular uptake and access to the vitamin D receptor. It is therefore important to elucidate the possible role played by the binding or lack of binding to DBP *in vivo*. We have investigated the relationship between the binding affinity for human DBP and the serum level and serum half-life ($t_{1/2}$) in rats of a series of new vitamin D analogues. The binding affinity for DBP was determined by displacement of ^3H-$1,25(OH)_2D_3$ from DBP attached to Affi-Gel 10. The serum levels in rats following a single intravenous dose were assessed by HPLC and the serum half-life was determined for each analogue. In the group of vitamin D analogues which showed a low or no affinity for DBP, we have identified compounds with a short $t_{1/2}$ and compounds with a long $t_{1/2}$, all characterized by low initial serum levels. Compounds with a long $t_{1/2}$ were also found in the group with a high affinity for DBP, and they were easily identifiable by their high initial serum level. These results showed that the initial serum level of vitamin D analogues correlated with the affinity for DBP, but that there seemed to be no correlation with the metabolic rate as reflected by measurement of the serum half-life of the analogues.

Keywords: $1\alpha,25(OH)_2D_3$; vitamin D analogues; pharmacokinetics; DBP

Introduction

$1\alpha,25(OH)_2D_3$, the physiologically active form of vitamin D_3, plays a crucial role both in the regulation of calcium homeostasis (DeLuca *et al.*, 1990) and in cell growth regulation (Reichel *et al.*, 1989). $1\alpha,25(OH)_2D_3$, vitamin D_3 and $25OHD_3$ are bound in the serum to a specific carrier protein, DBP (Haddad & Walgate, 1976; Bouillon *et al.*, 1981). The biological role of this protein is not clearly understood (Constans, 1992), but it has been suggested that binding to DBP protects $1,25(OH)_2D_3$ and its analogues from rapid metabolic degradation (Bouillon *et al.*, 1991; Dusso *et al.*, 1991).

The clinical usefulness of $1\alpha,25(OH)_2D_3$ is mainly limited by its effects on calcium metabolism. However, new vitamin D analogues with strong antiproliferative effects but with reduced effects on calcium metabolism have recently been described (Binderup & Bramm, 1988; Binderup *et al.*, 1991a,b; Hamada & Shinomiya, 1993). The study of these compounds has shown that most of them bind to DBP with a low binding affinity (Bouillon *et al.*, 1991; Dusso *et al.*, 1991). It has also been shown that vitamin D analogues with a low binding affinity for DBP, when tested *in vitro* in the presence of serum which contains DBP, have relatively high biological activities compared to $1\alpha,25(OH)_2D_3$, as the unbound analogues are able to enter the cells more freely (Vanham *et al.*, 1988; Bouillon *et al.*, 1991; Dilworth *et al.*, 1994).

In the search for new vitamin D analogues as candidates for the treatment of diseases such as cancer or autoimmune disorders, it is important to elucidate the pharmacokinetic profile of each compound. As it has previously been suggested that a low DBP affinity leads to a rapid degradation (Bouillon *et al.*, 1991; Dusso *et al.*, 1991), we found it of interest to investigate the relationship between the affinity for DBP of a series of vitamin D analogues and their pharmacokinetic characteristics, such as their serum level and metabolic rate, as reflected by measurement of their serum half-life in rats.

Results

Table 1 shows the binding affinity for human DBP of a new series of vitamin D analogues and their relative binding affinity compared to $1\alpha,25(OH)_2D_3$ in each experiment. The structures of the analogues are shown in Figure 1. In accordance with previous reports, it was found that $25OHD_3$ bound to DBP with a higher binding affinity than $1\alpha,25(OH)_2D_3$ (Dusso *et al.*, 1991;

Table 1 The affinity for DBP of vitamin D analogues: binding to purified human DBP (50% displacement)

Compound	*Binding affinity for DBP (M)*	*Relative binding affinity for DBP**
$25OHD_3$	9×10^{-9}	33
$1\beta,25(OH)_2D_3$	1.7×10^{-8}	17
$1\alpha,25(OH)_2D_3$	$1.5\text{-}6.0 \times 10^{-7}$	1
Calcipotriol	1.5×10^{-6}	0.1
MC 1127	5.2×10^{-6}	0.1
EB 1089	7.9×10^{-6}	0.03
CB 966	3.2×10^{-5}	0.02
KH 1139	6.5×10^{-5}	0.007
KH 1060	n.b.	0
KH 1049	n.b.	0

n.b. = no binding. *, The relative numbers are correlated to the $1\alpha,25(OH)_2D_3$ value obtained in each experiment

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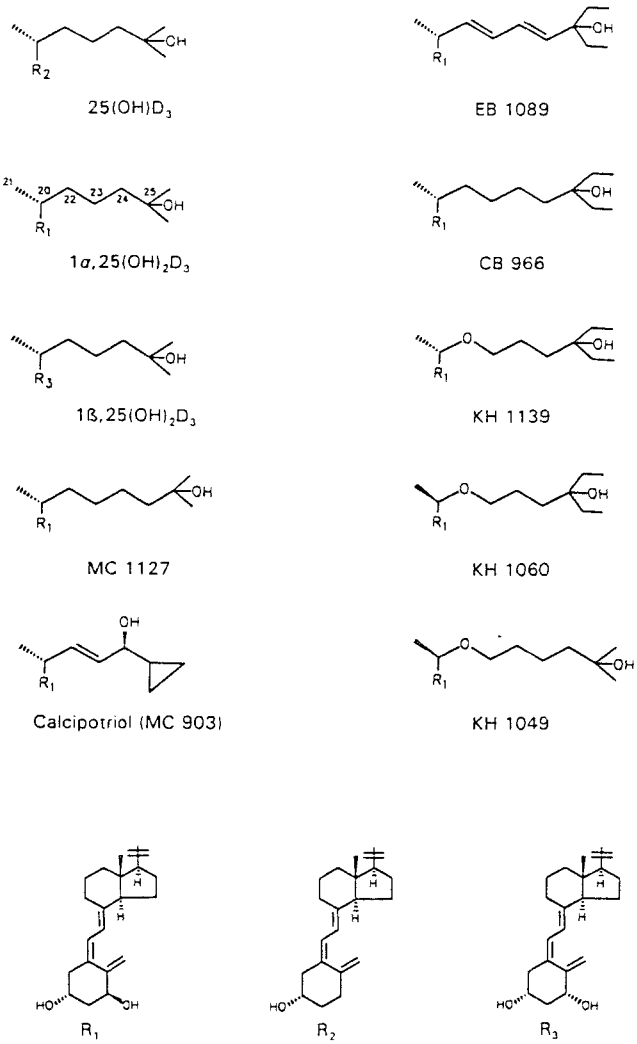

Figure 1 Chemical structures of $1\alpha,25(OH)_2D_3$, $25OHD_3$ and new vitamin D analogues. R_1, R_2 and R_3 represent different vitamin D ring systems

Bouillon *et al.*, 1992), the relative binding affinity being 33 times higher than that of $1\alpha,25(OH)_2D_3$. $1\beta,25(OH)_2D_3$ has also been described to bind to DBP with a high affinity (Bishop *et al.*, 1994). In our investigations the affinity of $1\beta,25(OH)_2D_3$ was approximately 17 times higher than that of $1\alpha,25(OH)_2D_3$. All other analogues tested bound to DBP with a lower binding affinity than $1\alpha,25(OH)_2D_3$. Of these, MC 1127 and calcipotriol showed the highest binding affinity. The compounds KH 1139, KH 1060 and KH 1049 did not show any binding to DBP when tested in concentrations up to 10^{-4} M.

In order to investigate the correlation between the pharmacokinetics of the vitamin D analogues and their affinity for DBP, the initial serum concentration ($C_{t=5\,min}$), the serum half-life ($t_{1/2}$), the area under the serum level/time curve (AUC∞) and the serum clearance were calculated from the serum concentrations determined by HPLC (Table 2). The initial serum levels of $25OHD_3$, $1\alpha,25(OH)_2D_3$ and $1\beta,25(OH)_2D_3$ were very high. However, the serum level of most of the other compounds was very low, with the exception of MC 1127, which had a relatively high serum level.

The serum $t_{1/2}$ was longer than 3–4 h for $25OHD_3$ and $1\beta,25(OH)_2D_3$, whereas the $t_{1/2}$ for $1\alpha,25(OH)_2D_3$, MC 1127, EB 1089 and CB 966 was approximately 2 h. The rest of the compounds had a $t_{1/2}$ shorter than 1 h. The AUC∞ indicates the concentration and persistence of the drug in the serum and it is therefore considered as a measure for the availability of the drug. The serum clearance is the volume of serum that is cleared totally of the drug per time unit, and it varies inversely with the AUC∞, as it can be calculated from the dose divided by AUC∞. Both the clearance and the AUC values are related to the volume of distribution of the drug and to its elimination rate. $25OHD_3$, $1\alpha,25(OH)_2D_3$ and $1\beta,25(OH)_2D$ have been shown to have a long $t_{1/2}$ combined with a high initial serum level, resulting in a high AUC∞ value and a slow clearance. KH 1139, KH 1060, KH 1049 and calcipotriol are compounds with a short half-life combined with a low initial serum level resulting in a low AUC and a fast clearance. EB 1089 and CB 966 are examples of compounds with a long $t_{1/2}$ but with a low initial serum level, also resulting in a relatively low AUC∞ value and a fast clearance. Both a relatively long $t_{1/2}$ and a relatively high initial serum level were seen with MC 1127, resulting in an AUC∞ that was higher than that of most of the compounds, and in a relatively slow clearance (Table 2 and 3).

Discussion

The object of the present investigation has been to study the relationship between the affinity for DBP, the major carrier protein for vitamin D_3 and its metabolites in the serum, and the pharmacokinetic parameters of a series of new vitamin D analogues. The analogues MC 1127, EB 1089, CB 966, KH 1139, KH 1060 and KH 1049 have been selected as interesting candidates for further studies in animals and humans (Binderup *et al.*, 1991a,b). These analogues are characterized by structural modifications in the side chain of the sterol molecule and a number of such modifications have previously been shown to result in a lower affinity for DBP (Bouillon *et al.*, 1991; Dusso *et al.*, 1991). Two vitamin D compounds $25OHD_3$ and $1\beta,25(OH)_2D_3$, which have higher binding affinities for DBP than $1\alpha,25(OH)_2D_3$ (Bishop *et al.*, 1994) have also been included in the study, together with the antipsoriatic analogue calcipotriol, which has previously been shown to have a reduced binding affinity for DBP (Bouillon *et al.*, 1991).

The previous studies (Bouillon *et al.*, 1991; Dusso *et al.*, 1991) have examined the binding affinities of various analogues for rat DBP and human DBP, respectively. No species differences were observed between the two studies. In the present investigation, human DBP was used, due to its high degree of purification. In analogy to the previous studies, $25OHD_3$ and $1\beta,25(OH)_2D_3$ were found to bind with high affinity to DBP. All the other analogues had lower affinities than $1\alpha,25(OH)_2D_3$, with MC 1127 and calcipotriol having the relatively highest binding affinities.

In addition to DBP studies, the pharmacokinetic profile of the compounds was also investigated. By comparing the binding affinities of vitamin D ana-

Table 2 Pharmacokinetic data on vitamin D compounds following a single intravenous dose of 200 μg/kg to rats

Compound	*Serum conc. at t = 5 min ng/ml*	$t_{1/2}$ *hours*	*AUC∞ ng/ml × h*	*Serum clearance ml/h/kg*
$25OHD_3$	2040	>2.8	9596	21
$1\alpha,25(OH)_2D_3$	2429	2.2	7355	27
$1\beta,25(OH)_2D_3$	2912	>4	13228	15
Calcipotriol	121	0.2	27	7407
MC 1127	545	1.6	1216	167
EB 1089	152	2.1	255	784
CB 966	176	1.8	267	693
KH 1139	154	0.7	142	1408
KH 1060	103	0.4	46	4348
KH 1049	104	0.5	40	5000

Table 3 The correlation between DBP affinity, initial serum concentration and serum half-life

	High initial serum level long $t_{1/2}$	*Low initial serum level short* $t_{1/2}$	*long* $t_{1/2}$
High DBP affinity	$25OHD_3$ $1\alpha,25(OH)_2D_3$ $1\beta,25(OH)_2D_3$ MC 1127		
Low or no DBP affinity		KH 1139 KH 1060 KH 1049	EB 1089 CB 966

logues shown in Table 1 with the values for the serum clearance in Table 2, it appears that these two parameters are correlated. The clearance is dependent on the volume of distribution (or initial serum level) and the elimination half-life. From Table 3 it can be seen that there is a strong correlation between the affinity for DBP and the initial serum level of the analogues, as all compounds with a high initial serum level have a high affinity for DBP, and all compounds with a low initial serum level have a very low or no affinity for DBP. These findings suggest that the affinity of the analogues for DBP exerts an influence on their serum level or the volume of distribution, which again influences the clearance of the compounds. However, no relationship between the affinity for DBP and the metabolic elimination rate of the compounds was observed, as exemplified by the existence of compounds with a low or no affinity for DBP, but with either a long or a short serum half-life. Therefore, our results do not support the theory that vitamin D analogues with a low binding affinity for DBP are more rapidly degraded than $1\alpha,25(OH)_2D_3$ (Bouillon *et al.*, 1991; Dusso *et al.*, 1991). EB 1089 and CB 966 are typical examples of compounds with a low binding to DBP, but with a slow metabolic degradation rate.

In the present investigation calcipotriol emerges as the only atypical analogue. Despite its having a relatively high affinity for DBP, the initial serum level of calcipotriol is very low. This may be due to the extremely fast rate of metabolic degradation that occurs with calcipotriol (Kissmeyer & Binderup, 1991). At the first sampling time, 5 min after dosing, extensive metabolism has already occurred.

In summary, it has been demonstrated that the serum levels obtained after systemic administration to rats of a series of vitamin D analogues are closely correlated with their affinity for DBP. In contrast, the serum half-life of each compound is an independent parameter that may become very important in the selection of new analogues for clinical investigation. A short half-life is desirable for compounds for topical use. In the search for compounds for systemic administration, a longer half-life is needed, as is the case of EB 1089, which is presently under investigation in patients with breast cancer.

Materials and methods

Compounds

$25OHD_3$, $1\alpha,25(OH)_2D_3$, $1\beta,25(OH)_2D_3$, calcipotriol (MC 903), MC 1127, EB 1089, CB 966, KH 1139, KH 1060 and KH 1049 were synthesized in the Department of Chemical Research, Leo Pharmaceutical Products. The compounds were dissolved in 2-propanol and stored at −20°C. For the *in vivo* experiments the compounds were diluted prior to administration with a vehicle suitable for i.v. administration: Citric acid 0.16 mg, trisodium citrate 6.8 mg, ethanol 80 mg, propylene glycol 415 mg and water up to 1.0 ml.

All other chemicals and reagents were commercially available.

DBP assay

DBP-Affi-Gel To improve the separation of bound and free vitamin D analogue, DBP was attached to a bed support (Affi-Gel 10, Bio-Rad Laboratories, Copenhagen, Denmark), by covalent linkage of the N-terminal end of the protein. Affi-Gel 10 was activated by repeated washing with 10 mM sodium acetate, pH 4.5 and adjusted to pH 8.5 by rinsing with 0.1 M $NaHCO_3$. Purified human DBP (Biodesign, AH Diagnostics, Aarhus, Denmark, >95% pure) was solubilized at 2.5 mg/10 ml in 0.1 M $NaHCO_3$, pH 8.5 and added to the pellet obtained from 25 ml Affi-Gel 10. The total volume was adjusted to 37.5 ml with 0.1 M $NaHCO_3$, pH 8.5, and the DBP-Affi-Gel was kept overnight at 4°C to allow for attachment to the bed support. Free binding sites were blocked by addition of glycine ethyl ester (final concentration of 0.1 M) for 1 h at 4°C. The DBP-Affi-Gel was washed twice with 0.1 M $NaHCO_3$ and stored at 4°C in 30 mM barbital buffer, pH 8.6 (binding buffer).

DBP binding assay The displacement of $[^3H]$-$1\alpha,25(OH)_2D_3$ from DBP-Affi-Gel was measured after addition of non-radioactive $1\alpha,25(OH)_2D_3$ or analogue. The compounds were tested at concentrations from 10^{-9}–10^{-4} M. Each sample contained 25 μl of $[^3H]$-$1\alpha,25(OH)_2D_3$ (Amersham, Birkerod, Denmark) in a final concentration of 2×10^{-7} M (app. 170 Bq), 250 μl of DBP-Affi-Gel, the vitamin D analogue dissolved in 25 μl of isopropanol and 200 μl of binding buffer. Affi-Gel 10 without attached DBP was used as blank. The samples were incubated for 2 h at room temperature

under constant stirring. The fraction of bound compound was separated from the free compound by repeated washing with binding buffer. The fraction of bound compound was counted with a β-counter. Each analogue was tested in duplicate experiments.

Pharmacokinetic experiments

Animals Groups of three male Sprague Dawley rats (150–250 g) were given a single intravenous dose of 200 μg/kg of test compound. This high dose was chosen as a compromise between a tolerable dose and a dose that would give measurable serum concentrations of the test compound. Blood samples were taken at 0, 5, 15 and 30 min, and at 1, 2 and 4 h after administration from different groups of animals each time, and serum was prepared. The animal studies were conducted in accordance with the Danish Animal Welfare Act.

Analysis The concentration of the parent compound was determined by HPLC. The HPLC system consisted of a Merck-Hitachi L-6200 Intelligent Pump, a Spark-Holland Marathon autosampler, a Spark-Holland Prospekt automated sample processor, a Merck-Hitachi L-4200 UV-VIS Detector and a Merck-Hitachi D-2500 Chromato-Integrator. The analytical column was a Merck LiChrospher 100 RP-18.5 μm, 125 × 4 mm column. The eluent was a linear water-methanol gradient from 70% to 95% methanol over 20 min. The wave length of detection was 264 nm. The sample preparation was as follows: The internal standard ($1,25(OH)_2D_3$ or an analogue with an appropriate retention time close to the test compound) was added to the serum, which was then precipitated with one volume of acetonitrile before 1.1 ml of the supernatant was diluted with 0.6 ml 0.01 M $(NH_4)_2HPO_4$ and 1.3 ml of this mixture was injected into the Prospekt automated sample processor, using a Marathon autosampler. In the Prospekt, a C8 cartridge from Analytichem was used for the sample enrichment. The cartridge was prewashed online with approx. 1 ml MeOH and 2 ml 0.01 M $(NH_4)_2HPO_4$ buffer, before the sample was loaded onto the cartridge. The cartridge was then washed with approximately 0.5 ml MeOH-H_2O (1:1) before the sample was eluted to the analytical column. The limit of detection was 10 ng per ml serum, and did not allow for detection of any metabolites.

Calculations The serum half-life ($t_{1/2} = \ln 2/ke$) was calculated from the elimination rate constant (ke), which was determined from linear regression of ln (conc.) *vs.* time. The area under the serum level/time curve (AUC∞) was calculated by the trapezoidal rule. The serum clearance was calculated by dividing the dose per kg by the AUC∞.

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