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# 1,25-Dihydroxyvitamin $D_3$ in lipiodol for the treatment of hepatocellular carcinoma: cellular, animal and clinical studies☆

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**Abstract**

1,25-Dihydroxyvitamin $D_3$ (1,25-$(OH)_2$ $D_3$) is a potent regulator of cell growth and differentiation, with recent evidence showing inhibition of tumor invasion, angiogenesis and tumor cell death. The growth–inhibitory properties of 1,25-$(OH)_2$ $D_3$ could be harnessed in the treatment of patients with cancer if the development of systemic hypercalcemia is avoided. Hepatocellular cancer (HCC) presents a setting where the tumor is accessible for treatment through the hepatic artery and also where the tumor is highly lipiodol avid. On this basis, we hypothesised that, 1,25-$(OH)_2$ $D_3$ dissolved in lipiodol and administered through the hepatic artery may prove to be a rational approach to the use of the drug in the treatment of HCCs. In brief, 6 years of work with 1,25-$(OH)_2$ $D_3$ at cellular, animal and clinical level has provided us with plenty of support for this hypothesis. Sensitivity of HCCs in cell culture to 1,25-$(OH)_2$ $D_3$, growth retardation of human HCC xenografts in nude mice, uptake and retention of 1,25-$(OH)_2$ $D_3$–lipiodol by liver tumors in cell culture and animals, escalation of the 1,25-$(OH)_2$ $D_3$ dose by 100× without the development of hypercalcemia in both liver tumor bearing rats and in patients with HCC are some of the evidence that will be discussed in this paper.


*Keywords:* 1,25-Dihydroxyvitamin $D_3$; Lipiodol; Hepatocellular carcinoma

## 1. Introduction

Since the first observation by Eisman of the expression of Vitamin $D_3$ receptors in cultured human breast cancer cells [1], the relationship between this hormone and cancer became the subject of immense research. The first evidence for a direct growth regulating effect of 1,25-dihydroxyvitamin $D_3$ (1,25-$(OH)_2$ $D_3$) on cancer cells emerged in 1981 when Colston et al. demonstrated the ability of the hormone to inhibit the growth of cultured melanoma cells at nanomolar concentrations [2]. At the same time Abe et al. reported differentiation of mouse myeloid leukemia cells by 1,25-$(OH)_2$ $D_3$ [3]. Today 25 years after the Eisman report, there is several lines of evidence to support an anticancer effect for 1,25-$(OH)_2$ $D_3$ in several systemic cancers. Hepatocellular cancer (HCC) is a common cancer worldwide for which most patients present at a stage in which surgical resection is no longer possible; the prognosis is dismal with very low response rates to chemotherapy and no or modest improvement in survival [4]. This together with the theory that intra-hepatic arterial (IHA) administration of very lipid soluble antiproliferative agents lacking hepato-toxicity but with a high degree of first pass effect, may prove to be useful agents in the treatment of HCC, led to the design of the following study.

## 2. 1,25-$(OH)_2$ $D_3$ inhibits in vitro HCC cell proliferation

Using different techniques of monitoring cell replication, several human (SKHEP-1, PLC/PRF-5, HepG2, and Hep 3β) and rat (Novikoff and HTC) HCC cell lines were examined for sensitivity to 1,25-$(OH)_2$ $D_3$. Five days of treatment with various concentrations ($10^{-11}$ to $10^{-6}$ μM) of 1,25-$(OH)_2$ $D_3$, led to dose and time dependent inhibition of proliferation. Here, HepG2 and Hep 3β exhibited very high sensitivities to the inhibitory effects of 1,25-$(OH)_2$ $D_3$. Whereas, PLC/PRF/5, SKHEP-1 and the rat cell line HTC exhibited low to modest sensitivity, Novikoff the other rat HCC cell line was almost totally resistant [5]. The two cell lines, HepG2 and Hep 3β with $IC_{50}$ values of less than 1 ηM, are probably some of the most sensitive cancer cell lines to the effect of 1,25-$(OH)_2$ $D_3$ reported so far. Clonal growth of HepG2 cells in response to 1,25-$(OH)_2$ $D_3$ was also dose

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dependent [6]. Treatment of HepG2 cells with 1,25-$(OH)_2$ $D_3$ analogues EB 1089 and CB 1093 also led to profound inhibition of proliferation of these cells. EB 1089 proved to be the most potent of the three compounds tested. Removal of the drug from the medium led to recovery of cells indicating that the 1,25-$(OH)_2$ $D_3$ (or analogues) induced inhibition is of a reversible type [7]. How 1,25-$(OH)_2$ $D_3$ inhibits proliferation of malignant cells has been the subject of much research in the last two decades and will be briefly discussed in relevant parts of this review.

## 3. Vitamin D receptor (VDR) in HCCs

The classical signaling pathway of 1,25-$(OH)_2$ $D_3$ employs the nuclear VDR which is transcription factor for 1,25-$(OH)_2$ $D_3$ target genes [8]. In an attempt to investigate the mechanism by which 1,25-$(OH)_2$ $D_3$ inhibits HCC cell proliferation, presence of VDR in HCC cells were examined [6]. Levels of functional VDR in HepG2 cells were determined and compared to a number of liver cancer cell lines including Novikoff which we had already shown to be resistant to the antiproliferative effects of 1,25-$(OH)_2$ $D_3$ [5]. While, the highest concentration of VDR determined by radio-ligand binding assay was found in HepG2 cells, Novikoff cells contained no detectable VDR by this method. Other liver cancer cell lines in which the antiproliferative effects of 1,25-dihydroxyvitamin $D_3$ were less profound than in HepG2 were also found to have lower levels of VDR than HepG2. Expression of VDR was further investigated by reverse transcription-PCR amplification of VDR cDNA from each cell line. The results of which, suggested that the lack of functional VDR receptor in Novikoff is due to lack of VDR gene expression and that the differential antiproliferative effects of 1,25-$(OH)_2$ $D_3$ seen with this cell line and the more sensitive liver cancer cell lines, especially HepG2, was due to differential expression of VDR [6]. Our results demonstrated that these cell lines express functional receptors able to specifically bind $^3$H-1,25-$(OH)_2$ $D_3$. In line with earlier reports, these results suggested that the antiproliferative effects of 1,25-$(OH)_2$ $D_3$ correlate well with the level of VDR within the cell.

## 4. 1,25-$(OH)_2$ $D_3$ induced cell cycle arrest

Further work in HepG2 cells, revealed that the growth inhibitory effect of 1,25-$(OH)_2$ $D_3$ probably results from arrest of cells in the G0/G1 phase of the cell cycle. Increases in the fraction of cells in G0/G1 were dependent upon the concentration of 1,25-$(OH)_2$ $D_3$ and were accompanied by complementary decreases in the number of cells in S phase. There was no change in the number of cells in G2 − M at any concentration of the hormone employed in our study [6]. Cell cycle arrest by 1,25-$(OH)_2$ $D_3$ has also been documented in a number of cancer cell lines of diverse origin including pancreatic [9], myeloid [10], prostate [11] breast [12], and myeloma [13]. Generally cells are arrested by 1,25-$(OH)_2$ $D_3$ in the G0/G1 phase although an additional block in G2 + M has been reported in one human breast cancer cell line at concentrations over $10^{-7}$ M and in the human promyelocytic leukemia cell line HL60 [10]. Treatment of HepG2 cells with 1,25-$(OH)_2$ $D_3$ led to the expected arrest in G0/G1 without any detectable block in other phases of the cell cycle even at the highest concentration of 1,25-$(OH)_2$ $D_3$ used. Based on results obtained in a number of different cancer cell types it has been postulated that blocking of the G1 phase by 1,25-$(OH)_2$ $D_3$ is accompanied by decreased cyclin C and D1 expression and increased expression of cyclin-dependent kinase inhibitors p21 and p27 [14].

## 5. 1,25-$(OH)_2$ $D_3$ retards HCC xenograft in nude mice

Next the effect of 1,25-$(OH)_2$ $D_3$ on the rate of growth of human HCC (SKHEP-1) xenografts in nude mice was investigated. Treatment of these animals with different doses (0.02–0.5 μg/kg per day) of 1,25-$(OH)_2$ $D_3$ significantly retarded tumor growth in the group receiving the highest dose (0.5 μg/kg). In these animals and at the doses employed, daily 1,25-$(OH)_2$ $D_3$ treatment for 21 days did not induce significant increase in serum calcium levels. For this study, noncalcaemic doses of 1,25-$(OH)_2$ $D_3$ were chosen on the basis of data obtained in previous pilot studies conducted in our labs and those reported by other researchers [15–17]. 1,25-$(OH)_2$ $D_3$ and its analogues have been shown to inhibit tumor growth in melanoma [15], breast [15,16,18], prostate [19], colon [17], lung [13] and squamous cell [20,21] carcinoma animal tumor models.

## 6. 1,25-$(OH)_2$ $D_3$ undergoes extensive first pass metabolism in the liver

The clinical use of 1,25-$(OH)_2$ $D_3$ and analogues has been hampered by the development of hypercalcemia upon systemic administration. 1,25-$(OH)_2$ $D_3$ is extensively metabolized in the liver accomplished via several catabolic routes, with 24-hydroxylation initiating the apparent pathway for the elimination of the hormone [22–24]. We hypothesised that a clinically significant hepatic first pass effect may exist upon the administration of 1,25-$(OH)_2$ $D_3$ as a hepatic arterial infusion, and that such an effect may allow high levels of 1,25-$(OH)_2$ $D_3$ to be delivered to the liver and consequently the liver tumor whilst avoiding high systemic levels. To examine this hypothesis two groups of landrace pigs were given identical doses of 1,25-$(OH)_2$ $D_3$ as continuous infusions, one group systemically, the other as a hepatic arterial infusion. Serum levels of 1, 25-$(OH)_2$ $D_3$, calcium, phosphate and a number of liver and kidney function tests were performed regularly. Concentrations of 1, 25-$(OH)_2$ $D_3$ and calcium remained normal in the hepatic arterial infusion an-

imals, in contrast to the intravenous infusion animals which developed elevated levels of 1,25-$(OH)_2$ $D_3$ and hypercalcemia [25]. Hepatic arterial infusion of 1,25-$(OH)_2$ $D_3$ did not produce any adverse effects upon renal nor hepatic function. These findings provided support for the idea that hepatic arterial infusion of 1,25-$(OH)_2$ $D_3$ may have potential in the treatment of hepatic cancers.

## 7. IHA administration of 1,25-$(OH)_2$ $D_3$ in patients with liver cancers

Results from the above study revealed that 1,25-$(OH)_2$ $D_3$ undergoes extensive first pass metabolism in the liver. This suggested that hepatic regional administration might allow high doses of 1,25-$(OH)_2$ $D_3$ to be administered for the treatment of liver cancers without producing hypercalcemia. A phase I study was then designed to investigate the effect of IHA administration of 1,25-$(OH)_2$ $D_3$ on serum calcium levels, together with other markers of liver and kidney function. Six subjects with hepatic colorectal cancer metastases and one with primary hepatocellular cancer were given continuous arterial infusions of 1,25-$(OH)_2$ $D_3$ for periods of 1–4 weeks. No patient developed hypercalcemia during the treatment at doses up to and including 10 μg per day. However, two out of the three patients given 15 μg per day became hypercalcemic. Except for one patient all others remained within the normal blood phosphate level [26]. These results revealed that when given through the hepatic artery, the 1,25-$(OH)_2$ $D_3$ dose can probably be raised up to 10 μg per day. Results from a phase I trial by Smith et al. [27] show that, subcutaneous injection of 10 μg 1,25-$(OH)_2$ $D_3$ every other day has led to the development of hypercalcemia in all patients. Similarly, in another phase I clinical trial when EB 1089 (the less calcemic analogue of 1,25-$(OH)_2$ $D_3$) was given to 36 patients with advanced breast and colorectal cancer in doses of between 0.15 and 17.0 μg/$m^2$ per day, hypercalcemia was seen in all patients receiving the 17.0 μg/$m^2$ per day dose [28].

In the last 20 years intra-hepatic arterial administration of drugs has drawn significant attention in the treatment of HCCs. Besides the favorable tumor blood supply, it has been shown that HCCs are very lipid avid. This has led to the development of the idea of using lipids and lipiodol in particular for the delivery of chemotherapeutic agents.

## 8. 1,25-$(OH)_2$ $D_3$ dissolved in lipiodol

In the chemotherapy of patients with HCC and in an attempt to increase drug efficacy and reduce systemic toxicity, regional delivery of drugs through the hepatic artery has been described. Liver tumors have been shown by many authors to have a predominantly hepatic arterial rather than portal venous blood supply [29–31]. Naturally, this route of administration enables the achievement of higher drug concentrations at the tumor site, while reducing exposure of rest of the body to higher drug concentrations and potentially toxic effects. Several carriers for the selective delivery of cytotoxic drugs to liver tumors have been investigated, amongst which, lipiodol is considered to be the agent of choice for this purpose [32–36]. An iodinated derivative of poppy seed oil, lipiodol is avidly taken up and retained for long periods of time by 85% of HCCs, hence its widespread use in detection of HCCs by CT imaging [34,37–39]. When administered through the hepatic artery, lipiodol has been found to remain selectively in the neovasculature and extravascular tissues of HCCs for very long periods [35,40–42]. Consequently, in HCC, administration of anti-cancer drugs in lipiodol further increases the selectivity of drug administration and treatment. It has been shown that this leads to tumor drug concentrations that are several folds higher than plasma drug concentrations. Consequently, tumor cells are exposed to higher drug concentrations for much longer periods with reduced risk of causing systemic toxicity [34,43,44]. However, for this method to be applicable, the drug in question must be highly lipid soluble to allow favorable delivery, uptake and retention followed by sustained release from the lipid within the tumor. The secosteroid hormone 1,25-$(OH)_2$ $D_3$ is both very potent and highly lipophilic making it an ideal agent for delivery in lipiodol.

## 9. 1,25-$(OH)_2$ $D_3$–lipiodol is retained by HCCs

It was hypothesised that by dissolving 1,25-$(OH)_2$ $D_3$ in a lipid based carrier such as lipiodol and administering it as a hepatic arterial injection, it would be possible to achieve high local concentrations within HCCs for prolonged periods, whilst avoiding high systemic concentrations and hypercalcemia. This was examined by administering a hepatic arterial infusion of $C^{14}$ radiolabelled 1,25-$(OH)_2$ $D_3$ in either lipiodol, medium chain triglyceride (MCT) or saline to rats bearing Novikoff liver tumors. Animals were then sacrificed 1 h, 1 or 3 days post-drug treatment. Assay of serum and tissue concentrations revealed that at all three time points examined, concentration of 1,25-$(OH)_2$ $D_3$ was significantly higher in the tumor tissue of lipiodol and MCT treated rats compared to saline group. Assay of serum and tissue concentrations revealed that, this approach results in selective distribution of the drug into HCC tumor tissue while the systemic serum levels remain low. Moreover, 1,25-$(OH)_2$ $D_3$–lipid was found to be retained in the tumor for substantially longer period of time. Lipiodol was more effective in these respects than MCT [45].

## 10. Efficacy of 1,25-$(OH)_2$ $D_3$–lipiodol in cell culture

Based on these results, IHA administration of 1,25-$(OH)_2$ $D_3$ dissolved in lipiodol, may prove to be the way for selectively delivering high concentrations of the drug to lipiodol avid liver tumors without causing systemic side effects. This is because not only 1,25-$(OH)_2$ $D_3$ is a highly lipophilic

agent that easily dissolves in lipiodol but also because it is an extremely potent agent which means that low quantities need to be used. Moreover, it undergoes significant first pass metabolism in the liver thus reducing the chances of systemic adverse events. These characteristics make 1,25-$(OH)_2$ $D_3$ an ideal agent for IHA delivery in lipiodol. We therefore attempted to test the feasibility of IHA administered 1,25-$(OH)_2$ $D_3$–lipiodol in the treatment of liver cancers. In the first step, uptake of the oil by HepG2 cells were tested and confirmed. Microscopic examination of cells exposed to the lipiodol containing media revealed intra-cellular presence of the oil in abundance. Next, efficacy of the drug in lipid was tested in cell culture after acute and chronic treatment of cells. Treatment of cells with 1,25-$(OH)_2$ $D_3$ dissolved in lipiodol resulted in profound inhibition of cell proliferation even after a short exposure of cells to the drug confirming that 1,25-$(OH)_2$ $D_3$ dissolved in lipiodol probably acts as a sustained release drug depot within the cell [46]. Similarly dissolving 1,25-$(OH)_2$ $D_3$ in medium chain triglyceride resulted in sustained inhibition of proliferation of HepG2 cells long after the removal of the drug from the cell culture medium [47].

## 11. Maximum tolerated dose of IHA administered 1,25-$(OH)_2$ $D_3$–lipiodol in liver tumor bearing rats

At this stage it was necessary to work out the maximum tolerated dose of IHA administered 1,25-$(OH)_2$ $D_3$–lipiodol. This was done in rats with Novikoff liver tumors starting with the 10 μg dose of 1,25-$(OH)_2$ $D_3$ dissolved in 100 μl of lipiodol and later escalating the dose to 50, 100 and 200 μg/kg of the drug dissolved in the same volume (100 μl) of lipiodol. Analyses of the blood drawn from the tail vain just before drug administration and 5 days post-drug administration showed all calcium levels and liver function tests were within the normal range [48]. However, tumor volume measured prior to drug administration (V1) and then 5 days later at the time of euthanasia (V2) showed no regression. In vitro tests had already indicated that Novikoff is a resistant cell line to 1,25-$(OH)_2$ $D_3$. However, this study revealed that in rats with Novikoff liver tumors, IHA injection of up to 200 μg/kg doses of 1,25-$(OH)_2$ $D_3$ dissolved in lipiodol, does not lead to the development of hypercalcemia. Uptake, and subsequent sustained release of the drug from lipiodol within the tumor followed by rapid metabolism in the liver, might account for these observations. Subsequent to obtaining these results, a phase I clinical trial in patients with HCC was commenced [48].

## 12. Phase I clinical trial of IHA administration of 1,25-$(OH)_2$ $D_3$–lipiodol

The objective of this pilot clinical study was to evaluate the maximum tolerated dose of IHA administered 1,25-$(OH)_2$ $D_3$–lipiodol in patients with HCC.

Eight patients with refractory HCC were given a single intra-hepatic arterial dose (50 μg to first three, 75 μg to the next three and 100 μg to the last two subjects) of 1,25-$(OH)_2$ $D_3$ dissolved in 5 ml of lipiodol. Following this, for 4 weeks serum calcium, 1,25-$(OH)_2$ $D_3$, α-fetoprotein and a range of biochemical indices were measured. Results obtained revealed that, while in three patients the calcium levels exceeded the normal range, even at these extremely high doses of 1,25-$(OH)_2$ $D_3$, non of the patients developed grade 3 hypercalcemia. 1,25-$(OH)_2$ $D_3$ administration also led to transient stabilization of serum α-fetoprotein in some patients [49]. The data obtained support the hypothesis that, in patients with HCC, IHA delivery of 1,25-$(OH)_2$ $D_3$ in lipiodol can allow administration of supra-pharmacological doses of the drug with out the development of hypercalcemia. Further studies with IHA administered 1,25-$(OH)_2$ $D_3$–lipiodol is ongoing.

## 13. Conclusion

We have been able to show that 1,25-$(OH)_2$ $D_3$ and its analogues profoundly inhibit growth of HCCs under experimental conditions. In patients with HCC, delivery of 1,25-$(OH)_2$ $D_3$ in lipiodol through the hepatic artery enabled huge dose escalation with out the development of hypercalcemia. Further studies to optimize drug formulation and dosage are warranted.

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