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The pan-caspase inhibitor QVD has anti-leukemia effects and can interact with vitamin D analogs to increase HPK1 signaling in AML cells

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Abstract

Caspase function is known to be essential for cell death by apoptosis, but it is now increasingly recognized that these proteases also play important roles in other cellular events. Here we report for the first time that inhibition of cellular caspase activity can induce differentiation of AML blasts, and can enhance vitamin D-induced cell differentiation of these cells. This was studied in blasts obtained from nine patients with AML and one patient with CML by *ex vivo* culture in the presence of Q-VD-OPh (QVD), a pan caspase inhibitor. Cell differentiation was manifested by the expression of markers of monocytic differentiation CD11b and CD14. Differentiation induced by 1 α ,25-dihydroxyvitamin D (1,25D) or its analogs PRI-1906 and PRI-2191 was enhanced by QVD to a varying degree, depending on the subtype of the leukemia. QVD and 1,25D-induced differentiation was accompanied by increased signaling by Hematopoietic Progenitor Kinase 1 (HPK1), and the expression of transcription factors known to be involved in monocytic differentiation was increased. Although the magnitude and nature of these changes were not invariable, it is clear that caspase inhibitors warrant attention as components of differentiation therapy of leukemia, perhaps in combination with derivatives of vitamin D.

Keywords

Caspases; AML; CML; vitamin D; HPK1 pathway; Q-VD-OPh

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Contributions

XC performed research, analyzed data and wrote the paper; AK developed analogs and analyzed data; JSH provided the patients' specimens and analyzed data; GPS supervised the research, edited and approved the paper.

Conflict of Interest Statement

A.K. has financial interest in Pharmaceutical Research Institute, Warsaw, Poland and all other authors have no conflict of interest to declare.

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1. Introduction

One approach to targeted treatment of acute myeloid leukemia (AML) is differentiation therapy. For the last three decades, it has been known that $1\alpha,25$ -dihydroxyvitamin D₃ (1,25D) can induce monocytic differentiation of human AML blasts both *in vitro* and *ex vivo* [1]. However, the principal risks of the clinical use of 1,25D are the development of hypercalcemia or of leukemia resistance to 1,25D. While hypercalcemia can be a barrier to this approach, the use of 1,25D analogs such as PRI-1906, PRI-2191 and PRI-2201 (vitamin D derivatives, VDD) reduces this risk. In addition, combination of 1,25D with other agents such as the plant-derived antioxidant carnosic acid, and SB202190, a p38 kinase inhibitor, referred to as “DCS” cocktail, further increases differentiation potency of deltanoids in AML cells [2]. This enhancement of differentiation has been shown to be associated with activation of MAPK pathways [2], and most recently we found that the expression of full length hematopoietic progenitor kinase 1 (HPK1) protein and its downstream MAPK signaling are required for optimal induction of differentiation by 1,25D [3].

2. Patient characteristics

The study population was a cohort of 10 patients admitted to Robert Wood Johnson University Hospital New Brunswick, NJ, for management of either newly diagnosed, and as yet untreated AML (9 patients), or Chronic Myeloid Leukemia in Myeloid Blast Crisis (1 patient) who provided informed consent to obtain peripheral blood specimens. Table 1 summarizes the clinical pathologic evaluation of the patients performed as part of the routine management of these patients.

3. The intensity of monocytic differentiation induced by 1,25D and analogs in AML blasts varies with the FAB subtype and tends to parallel the expression of HPK1, an upstream MAP kinase

Following exposure to the differentiation protocols the cultured cells were examined by Flow Cytometry for myeloid/monocytic phenotype markers CD11b and CD14, as illustrated in Fig. 1A. The double-positive differentiated cell population (upper right quadrants) increased following treatment with 1,25D or analogs PRI-1906 or PRI-2101. This suggests a therapeutic advantage, as the PRI analogs were previously shown to be less calcemic than 1,25D [4]. To determine if HPK1 signaling is likely to contribute to the abrogation of the differentiation block characteristic of leukemogenesis, we found that HPK mRNA level increased in AML blasts treated by VDDs in primary culture, and in general, though not invariably, paralleled the expression of monocytic differentiation marker CD14 (Fig. 1B).

4. Caspase inhibitor QVD induces differentiation and increases HPK1 signaling

Since HPK1 signaling has been reported to be regulated by caspase cleavage [5, 6], we investigated whether this is the case in the human AML-differentiation system. Thus, we treated the *ex vivo* AML blasts with the potent pan-caspase inhibitor QVD [7], and found that QVD can potentiate differentiation induced by VDDs (Fig. 2). Surprisingly, QVD alone was also able to increase the expression of differentiation markers in these AML blasts, though to a varying degree, and this was also seen in other cases (Fig. 3). The interaction between caspase inhibition and VDD was however very complex, as in some cases VDDs inhibited the differentiation induced by QVD alone, although the effect of VDD/QVD combination was higher than VDD alone (Fig. 3A). In other cases QVD inhibited differentiation induced by VDDs (Fig. 3C), and in some cases there was mutual potentiation,

with the combination of QVD and VDD producing the highest levels of differentiation (Fig. 2).

The relationship between the differentiation effects of QVD and HPK-1 signaling was studied by determining the levels of the full length HPK-1 protein and the associated upregulation of its downstream kinase cascade. Protein levels of transcription factors (TFs) related to VDD-induced differentiation were also studied in three samples with sufficient material (Fig. 3). All three VDDs increased the expression of FL-HPK1, though with some variation between samples regarding the magnitude of the effects (Fig. 3A, B, C, lanes 1–4). Of note, the levels of P-JNK1, which is activated by HPK1 [3, 5], tended to parallel the expression of the monocytic surface marker CD14 (Fig. 3), and the TFs Jun-ATF2/AP1, C/EBP β , and Egr-1 followed this trend, further supporting a role of JNK1 in upregulation of TF which execute VDD-induced monocytic differentiation [2, 8].

5. QVD inhibits cleavage of HPK1

In an experiment utilizing 40AF cells, which model 1,25D-resistance in AML [2], we found that the abundance of the fragment of HPK1 protein cleaved from its C-terminal (HPK1-C), believed to be one of the factors responsible for the resistance to differentiation [3], is reduced when 1,25D resistance is reversed by DCS, while the FL-HPK1 protein shows increased level (Fig. 4A). When QVD was combined with DCS the expression of differentiation markers was higher than with DCS alone (Fig. 4B), as detailed in a recent report [3], and expression of FL-HPK1-c-Jun increased while HPK1-C level decreased when cells were treated by QVD or QVD/DCS (Fig. 4A). Because there was not enough material for additional experiments on cells *ex vivo*, we illustrate in Fig. 4, A and B a similar result in a model system provided by the 40AF AML cell line. This bolsters the *ex vivo* result shown in Fig. 4, C and D. Comparison of data shown in Fig. 4C with Fig. 4D suggests inhibition of HPK1 cleavage by QVD. It is apparent that QVD induced an increase of the levels of FL-HPK1 protein (Fig. 4C) even though HPK1 mRNA levels were markedly reduced (Fig. 4D). Thus, control of FL-HPK1 expression is clearly post-transcriptional, and at least in part, appears to occur by a proteolytic cleavage of the primary translational product.

6. Discussion

The non-apoptotic functions of caspases in cell differentiation are complex [9, 10], and appear dependent on the cell context. For instance, blocking of caspase activity during differentiation of primary mouse progenitor cells can lead to attenuated monocytic differentiation [5]. In contrast, inhibition of caspase activity in AML blasts in primary culture resulted in monocytic differentiation shown in Fig. 3. This suggests that caspase activity contributes to the block of differentiation in leukemic blasts.

We have observed that the effects of VDDs combined with QVD vary in different subtypes of AML blasts in primary culture. This is consistent with the known heterogeneity of mutations in AML. Of interest, blasts from the less mature subtype of leukemia M1 (acute myeloid leukemia without maturation), which have very low sensitivity to VDDs, were induced to differentiate by the VDD/QVD combination (Fig. 3A). On the other hand, a VDD alone can effectively induce differentiation in the more mature subtype M5b (acute monoblastic leukemia), so the addition of QVD appeared to be unnecessary to enhance the effect of VDDs in M5b blasts (Fig. 3C). VDDs alone have little effect on M1 cells but in these cells QVD can provide a stimulus required to differentiate by activating non VDR-dependent signaling pathways.

HPK1 was shown to activate the JNK pathway [5, 6], and JNK1 signaling has a positive role in differentiation of AML cells [2]. Here we find that the inhibition of caspase activity by

QVD can increase FL-HPK1-JNK1 signaling, and correlates with the increased expression of the downstream target C/EBP β (Fig. 3), one of the key TFs which activate monocytic differentiation [8]. One example is the M1 leukemia in our study which carries C/EBP α mutation (Fig. 3A). We propose that the FL-HPK1-JNK1-cJun-C/EBP β (and Egr-1) signaling cascade is promoted by VDD/QVD combination and bypasses the mutation-blocked C/EBP α pathway. Since approximately 15% of AML-M1 patients carry the C/EBP α mutation, the case we studied may be representative of the subgroup of patients in which C/EBP β upregulation by caspase inhibition can compensate for the loss of functional C/EBP α and permit differentiation.

Supporting these *ex vivo* results, we recently found that the expression of the full length HPK1 protein and its downstream MAPK signaling is required for optimal induction of differentiation by 1,25D and analogs in a variety of human AML cell lines [3]. This is consistent with the hypothesis that in human AML blasts caspases inhibit normal differentiation programs, at least in part, by a proteolytic cleavage of HPK-1.

Here we report for the first time that a caspase inhibitor can induce differentiation, as well as potentiate the effects of 1,25D or its analogs on differentiation of some subtypes of AML blasts. Generally, QVD is active at lower concentrations than the widely used apoptosis inhibitors, ZVAD-fmk and Boc-D-fmk, and is not toxic to cells even at very high concentrations [7].

Our results demonstrate the individual variation of AML blasts responses to selected agents with the ability, though not universal, to induce differentiation. Since AML is a very heterogeneous disease, with a whole gamut of mutations, this is not surprising. We propose that the experimental set-up we describe may provide a template for a simple test by which patients can be stratified for clinical trials of innovative differentiation therapy, to identify those who are likely to benefit from such treatment. Thus, our studies suggest further, more extensive, pre-clinical studies of QVD as a potential component of personalized anti-myeloid leukemia therapy.

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Fig. 1. Monocytic differentiation induced by 1,25D or analogs in AML blasts parallels the expression of HPK1

Patient blasts were exposed to 1,25D, or its analogs PRI-1906 and PRI-2191 (all at 10 nM), generally for 7 days, cells were harvested for experiments.

(A) An illustration of the induction of monocytic differentiation markers CD11b and CD14 in AML blasts by 1,25D, PRI-1906 or PRI-2191. (Flow cytometry, X axis = CD11b; Y axis = CD14). Percentages of double positive cells in right-upper quadrant are shown. (patient #6 AML-M5).

(B) CD14 expression detected by flow cytometry and HPK1 mRNA levels by RT-PCR in five patients' specimens. 1: Control; 2: 1,25D 10 nM; 3: PRI-1906 10 nM; 4: PRI-2191 10 nM.

Fig. 2. Caspase inhibitor QVD alone induces differentiation and when combined with VDDs enhances differentiation of AML blasts

Patient blasts were exposed to 1,25D, or its analogs PRI-1906 and PRI-2191 (all at 10 nM), and/or pan-caspase inhibitor QVD (5 μ M), generally for 7 days, cells were harvested for experiments.

(A) Representative images of flow cytometric analysis of AML-M2 (patient #9) blasts following exposure to QVD or 1,25D alone, or in combination. (X axis = CD11b; Y axis = CD14). Percentages of cells positive for a myeloid marker in each quadrant are shown.

(B) Summaries of differentiation results with four patients samples, which were treated by VDDs, QVD or VDD/QVD combination, obtained as illustrated in (A). 1: Control; 2: 1,25D 10 nM; 3: PRI-1906 10 nM; 4: PRI-2091 10 nM; 5: QVD 5 μ M; 6: QVD + 1,25D; 7: QVD + 1906; 8: QVD + 2191.

Fig. 3. QVD alone or combined with VDDs increases differentiation and HPK1-cJun signaling in AML cell context-dependent manner

(A) AML-M1 (patient #8) with C/EBPα mutation.

(B) AML-M2 (patient #4) with FLT-3 mutation.

(C) AML-M5 (patient #6), mutations not detected. Western blots and corresponding extent of differentiation are shown for three patients. Optical density, relative to loading control, is shown below each band. Hierarchical relationships of the signaling cascade are top to down. The expression of CD11b and CD14 was determined by flow cytometry. Experimental treatments are listed below, and are represented by the same numbers in all panels. (NS = a non specific band on the blot). 1: Control; 2: 1,25D 10 nM; 3: PRI-1906 10 nM; 4: PRI-2091 10 nM; 5: QVD 5 μM; 6: QVD + 1,25D; 7: QVD + 1906; 8: QVD + 2191.

Fig. 4. QVD increases the inhibition of cleavage of full-length HPK1, induced by differentiation enhancers, which correlates with c-Jun expression

(A) Inverse correlation of FL-HPK1/cJun pathway with the cleaved fragment of HPK1 (HPK1-C). Protein levels were detected by western blots in 1,25D-resistant 40AF cells after 72 hr treatment with DCS (10 nM 1,25D + 10 μ M carnosic acid + 5 μ M SB202190), QVD (5 μ M) or DCS/QVD.

(B) Representative images of flow cytometric analysis of differentiation markers in 40AF cells. (X axis = CD11b; Y axis = CD14). Percentages of cells positive for a myeloid marker in each quadrant are shown.

The contrast between QVD-induced increases in FL-HPK1 protein levels and reduction in HPK1 mRNA levels in two AML cases: (C) Patient #8; (D) Patient #4. The effect of QVD on FL-HPK1 protein on AML cells in primary culture was similar to its effect in 40AF cells shown in panel (A).

Table 1

Patient characteristics

No.	Age	Gender	FAB Dx	WHO Dx	Cytogenetics	Molecular Mutations
#8	34	M	AML-M1	Acute Myeloid Leukemia without maturation	46,XY	C/EBPa mutated
#2	69	F	AML-M2	Acute Myeloid Leukemia with maturation	46,XX	NPM-1 mutated
#9	48	M	AML-M2	Acute Myeloid Leukemia with maturation	46,XY t(8;21)	Not detected
#7	27	M	AML-M2	Acute Myeloid Leukemia with maturation	49,XY, +4,+8,+20	NPM-1 mutated
#4	50	M	AML-M2 with mixed lineage	Acute Myeloid Leukemia, biphenotypic	46,XY, t(6;14)	FLT-3 mutated, increased numbers of tandem repeats
#5	32	F	AML-M3	Acute Promyelocytic Leukemia	46,XX, t(15;17) (q22;q21)	RARα-PML chimera
#1	42	M	AML-M4	Acute Myelomonocytic Leukemia	46,XY	NPM-1 mutated
#3	46	F	AML-M5b	Acute Monoblastic Leukemia	46,XX, t(11;19) (q23;p13.3)	MLL-1 mutated at locus 11q23
#6	61	M	AML-M5b	Acute Monoblastic Leukemia	47,XY, +19	Not detected
#10	47	M	CML	Chronic Myelogenous Leukemia in myeloid blast crisis	46,XY, t(9;22), inv(11)	Not detected

Patient blasts were separated by Histopaque-1077 gradient centrifugation from freshly obtained patient's peripheral blood, then were exposed to 1,25D₃ or its analogs PRI-1906 and PRI-2191 (all at 10 nM), and/or pan-caspase inhibitor QVD (5 μM). After incubation at 37°C in RPMI 1640 with 10% bovine calf serum, generally for 7 days, cells were harvested for experiments. The duration of exposure depended on the time that the cells maintained their viability in culture. Some specimen had insufficient material to perform all studies. Each experiment represents one independent experiment using one AML patient's specimen at a time. The sequence is by French-American-British (FAB) subtype, and patient numbers refer to the order the patients presented. WHO = World Health Organization.