Cyclin D1 Overexpression Sensitizes Breast Cancer Cells to Fenretinide

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ABSTRACT

Purpose: Fenretinide has shown promise in the chemoprevention of breast cancer, a tumor type in which the oncogene cyclin D1 is overexpressed frequently. We aimed at determining the effect of cyclin D1 level on the response to fenretinide treatment.

Experimental Design: Stable clones of T47-D cells were created to overexpress cyclin D1 or a mutant of cyclin D1, injected in nude mice for xenograft formation, and the rate of tumor growth and tumor regression determined.

Results: We show here that cells overexpressing cyclin D1 are significantly more sensitive to fenretinide than genetically matched cells that express low levels of cyclin D1, and that fenretinide prevents tumor formation arising from cyclin D1-overexpressing cells. Furthermore, we show that fenretinide is also able to promote the regression of cyclin D1-positive tumors. We also show that cells expressing a mutant of cyclin D1 that cannot bind to cdk4 are also more sensitive to fenretinide.

Conclusions: These results suggest that fenretinide may be particularly useful in the treatment of cyclin D1-positive breast cancers, and that the interaction between cyclin D1 and fenretinide is independent of cyclin D1 binding to cdk4.

INTRODUCTION

One of the biggest challenges in oncology is to predict the individual cancer patient response to a particular treatment. Ultimately, establishing a correlation between the expression of one gene and response to a particular drug would be ideal. One stunning example of such a correlation is between the presence of the PML-RARα fusion protein in acute promyelocytic leu-

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kemia and treatment using ATRA3 (1). ATRA has been shown recently to also accelerate the degradation of cyclin D1 (2). Cdks are key cell cycle regulators, and their activities are modulated at several levels, including the binding to their regulatory subunits, the cyclins (3). Among the cyclin subunits, α-type cyclins associate with cdk4 and 6 to phosphorylate the Rb protein. Hyperphosphorylation of Rb promotes the release of the E2F family of transcription factors that then promotes entry into S phase through activation of key target genes (4). In human breast cancer, abnormal accumulation of cyclin D1 is observed in up to 35% of cases, and overexpression of cyclin D1 in mouse mammary glands leads to carcinoma (5, 6). Cyclin D1 is overexpressed by several mechanisms (7–9) and was shown recently to be essential for tumor formation in MMTV-erb-2 transgenic mice (10). The particular importance of cyclin D1 in breast cancer is additionally supported by the observation that cyclin D1 knockout mice show a defect in alveolar proliferation during pregnancy (11, 12).

Overexpression of cyclin D1 has been reported to promote sensitivity to retinoic acid in vitro (13). This result raises the interesting possibility that retinoic acid may be useful for the treatment of cyclin D1-overexpressing tumors. However, this study did not address the efficacy of ATRA against cyclin D1-overexpressing tumors in vivo and, furthermore, the use of ATRA against breast cancer is limited by its toxicity. Fenretinide is a synthetic analogue of ATRA, with several advantages over ATRA for the treatment of solid cancers including a low toxicity profile, a stable plasma concentration, and the ability to concentrate in the breast (14). Unlike ATRA, fenretinide induces apoptosis (15) and is a poor inducer of differentiation (16). The results of a clinical trial testing the efficacy of fenretinide in preventing the recurrence of breast cancer in early stage patients who had their primary removed surgically were reported recently. This trial indicated a potential benefit of fenretinide treatment in premenopausal women (17). However, because this trial was not targeted at a specific molecular marker, the optimal efficacy of fenretinide may not have been fully revealed. We initiated this study to test the effect of cyclin D1 overexpression on the response to fenretinide treatment in a xenograft model. We found that cyclin D1 overexpression sensitizes breast cancer cells to killing by fenretinide in vitro and in vivo. Our results raise the exciting possibility that fenretinide may be of particular benefit to the treatment of breast cancers characterized by the overexpression of cyclin D1.

3 The abbreviations used are: ATRA, all-trans retinoic acid; cdk, cyclin-dependent kinase; Rb, retinoblastoma; MMTV, mouse mammary tumor virus; C, control; P, parental; F, fenretinide; D, D1; CI, confidence interval.
TRANSMISSION OF GENETIC INFORMATION BY NA+–K+ ATPASE ACROSS THE INSULIN SECRETION BARRIERS.

Na+–K+ ATPase is a transmembrane protein that maintains cellular Na+ and K+ gradients and is essential for cell survival and function. In the context of insulin secretion, Na+–K+ ATPase is crucial for the maintenance of the plasma membrane potential and the regulation of intracellular calcium levels, which are essential for insulin release.

In the example of a溜溜球 tournament, Na+–K+ ATPase might be analogous to the tournament structure, where the participants (in this case, the Na+–K+ ATPase enzyme) are distributed across the various rounds, with each round representing a different concentration or condition. The exit of a participant from the tournament (i.e., the enzyme's ability to function under different conditions) can be affected by factors such as the concentration of a key substrate or inhibitor, which can influence the enzyme's activity and thus the outcome of the tournament.

The use of Na+–K+ ATPase in such a context highlights the enzyme's role in maintaining cellular homeostasis and its potential as a target for therapeutic intervention in diseases where these functions are impaired.

In summary, the role of Na+–K+ ATPase in insulin secretion is multifaceted, and understanding its regulation is crucial for the development of targeted therapies for diabetes and other metabolic disorders.
were determined by Western analysis using an anti-cyclin D1 antibody. Harvested and protein extracted. Levels of cyclin D1 and cyclin D1-HA in independent clones of T-47D cells stably expressing cyclin D1-HA were in vitro response to Fenretinide treatment overexpression of cyclin D1 promotes growth inhibition in Fig. 1A, proliferation, therefore 0% growth inhibition. The percentage of growth inhibition was determined. To calculate growth inhibition, cells were incubated in the presence or absence of 5 μM of fenretinide for 3, 4, or 5 days. Cells were harvested, fixed in 3.7% paraformaldehyde/PBS, and stained with 4',6-diamidino-2-phenylindole. The number of apoptotic nuclei in each population was determined. A total of 100 nuclei were counted in three separate experiments; bars, ±SD.

**Fenretinide Treatment Prevents Growth of Cyclin D1-overexpressing Xenograft.** To determine whether overexpression of cyclin D1 sensitizes cells to fenretinide treatment in vivo, we next used parental and D1 cells for the formation of xenografted tumors in nude mice. Mice were divided into four groups. Mice in group 1 were injected with P cells and were mock treated, and we refer to this group as the PC group. Mice in group 2 were injected with P cells and received F treatment (PF group). Mice in group 3 were injected with D cells and received mock treatment (DC group). Finally, mice in group 4 were injected with D cells and were treated with F (DF group). Tumor formation was monitored by weekly size measurements over a period of 9 weeks. Fenretinide or control treatments were initiated 1 day after cells were injected s.c. Whereas tumor growth was evident in PC (Fig. 2A), PF (Fig. 2B), and DC (Fig. 2C), tumor growth was clearly reduced in the DF group (Fig. 2D). The growth rate was defined as the percentage increase in tumor volume per week (±SE) and was estimated at 16% ± 2% for the PF group (Fig. 2E) in contrast to only 7% ± 1% in the DF group (Fig. 2F). Although statistical significance was not reached between DC group versus DF group (*P* = 0.13), statistical comparison between PF group (Fig. 2B) versus DF group (Fig. 2D) indicated a highly significant difference in tumor growth rate (*P* = 0.002). Fenretinide treatment did not affect the growth of the parental xenograft (PC versus PF; *P* = 0.6; Fig. 2, A and B). However, statistical comparison between the PC (Fig. 2A) and DC group (Fig. 2C) indicated a borderline significance (*P* = 0.043) suggesting that over a period of 9 weeks there is a possibility that the growth rate of D1 xenograft may be slower than that of parental xenograft, with a growth rate of 11% ± 2% compared with 17% ± 1%, respectively (Fig. 2E). However, result rules out the possibility that the effect observed is because of the HA tag. Western blotting revealed that fenretinide was able to reduce the levels of endogenous cyclin D1 protein in both parental and D1 cells (Fig. 1C) supporting the previous finding that fenretinide affects cyclin D1 transcription (18). Interestingly, exogenous cyclin D1-HA levels were also reduced by fenretinide, although the effect was modest, raising the possibility that fenretinide may also affect to some extent cyclin D1 proteolysis. In agreement with previous observations (18), we found no specific effect on cell cycle arrest after fenretinide treatment (data not shown). However, we found that overexpression of cyclin D1 increased the level of fenretinide-mediated apoptosis (Fig. 1D). These results show that overexpression of cyclin D1 leads to increased apoptosis after fenretinide treatment compared with genetically identical cells that do not express cyclin D1 at such elevated levels.

**Fig. 1** A, overexpression of cyclin D1 promotes growth inhibition in response to Fenretinide treatment in vitro. A, T-47D cells (P) and three independent clones of T-47D cells stably expressing cyclin D1-HA were harvested and protein extracted. Levels of cyclin D1 and cyclin D1-HA were determined by Western analysis using and anticyclin D1 antibody. B, T-47D (parental) and T-47D-D1 (D1 cells) cells (0.5 × 10⁶) were plated. The following day, fenretinide was added at increasing concentration. Three days after the addition of fenretinide, the percentage of growth inhibition was determined. To calculate growth inhibition, cells were counted, and cells that received no fenretinide were set to 100% proliferation, therefore 0% growth inhibition. The percentage of growth inhibition at each dose of fenretinide was calculated relative to the control population. Points are the mean of three separate experiments; bars, ±SD. C, parental and D1 cells were harvested after treatment with increasing dose of fenretinide and cyclin D1 levels determined by Western analysis using anticyclin D1 antibody. D, parental and D1 cells were incubated in the presence or absence of 5 μM of fenretinide for 3, 4, or 5 days. Cells were harvested, fixed in 3.7% paraformaldehyde/PBS, and stained with 4',6-diamidino-2-phenylindole. The number of apoptotic nuclei in each population was determined. A total of 100 nuclei were counted in three separate experiments; bars, ±SD.
this result needs to be interpreted with care, as a comparison of the two groups using the nonparametric Mann-Whitney test did not achieve statistical significance \( (P = 0.095) \). These observations suggest that fenretinide treatment reduces the rate of tumor formation arising from xenografts overexpressing cyclin D1, whereas such treatment had no effect on tumor formation arising from xenografts expressing low levels of cyclin D1.

**Fenretinide Treatment Promotes the Regression of Xenograft-overexpressing Cyclin D1.** As our results indicated a preventive effect of fenretinide on the progression of cyclin D1-overexpressing tumors, we next determined the effect of fenretinide on tumor regression. For this experiment, cells were injected and mice divided into four groups as described above. However, in contrast to the previous experiment, tumors were allowed to grow without any treatment for a period of 5 weeks. Statistical analysis of tumor growth between weeks 1 and 5 indicated that cyclin D1-overexpressing xenografts grew faster than the parental cells (Fig. 3; \( P = 0.023 \)). This result suggested that the overexpression of cyclin D1 promoted cellular proliferation during the initial establishment of tumor formation compared with an otherwise genetically identical cell population where cyclin D1 levels are low.

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**Fig. 2** Fenretinide treatment prevents growth of cyclin D1-overexpressing xenographs. A, at week 0, 10 nude mice were injected with parental cells (T-47D) and received mock treatment. Tumor growth was determined over a period of 9 weeks as described in “Materials and Methods.” B, 10 nude mice were injected with parental cells and treated with fenretinide. Tumor growth was determined over a period of 9 weeks. C, 10 nude mice were injected with D1 cells (T-47D-D1) and received mock treatment. Tumor growth was determined over a period of 9 weeks. D, 10 nude mice were injected with D1 cells and received fenretinide treatment. Tumor growth was determined over a period of 9 weeks. E, tumor growth rate for each group of mice. N indicates the number of mice per group remaining after 9 weeks. The mean and the 95% CI are shown below each group.

**Fig. 3** Measurement of tumor growth between weeks 1 and 5. Parental (T-47D) and D1 cells (T47-D-D1) were injected s.c. on the back of nude mice, and the tumor volume measured over a period of 5 weeks before any treatment. The tumor growth rate per week was determined in both groups. N indicates the number of mice per group remaining after 9 weeks. The mean and the 95% CI are shown below each group.
After 5 weeks, fenretinide treatment was initiated. Comparison of tumor growth from weeks 5 to 11 indicated that fenretinide treatment had no significant effect on the tumor growth between the parental groups (Fig. 4, A and B; \( P/H_11005 0.60 \)). However, fenretinide had a drastic effect on the growth of D1 tumors when compared with the mock-treated D1 tumors (\( P/H_11021 0.0005 \); Fig. 4, C and D). In addition, comparison between the PF group (Fig. 4B) and DF group (Fig. 4D) also indicated that fenretinide treatment lead to a drastic reduction in the growth of D1 xenografts compared with parental xenografts (\( P/H_11021 0.0005 \)).

The mean tumor growth rate for the DF group between weeks 5 and 11 (Fig. 4E) showed that fenretinide not only stopped the progression of D1 xenografts but also promoted tumor regression. Therefore, these results indicated that overexpression of cyclin D1 sensitizes cells to fenretinide in vivo.

Expression of Free Cyclin D1 Enhances the Sensitivity to Fenretinide. The role of cyclin D1 in complex with cdk4 is well known to promote cellular proliferation by leading to entry into S phase. However, several reports have described distinct roles for cyclin D1 independently of its binding to cdk4 that may contribute to its oncogenic action (19, 20). To determine whether the interaction between fenretinide treatment and cyclin D1 is dependent on its binding to cdk4, we created T47-D-based stable clones overexpressing a mutant form of cyclin D1 (cyclin D1-KE) that cannot bind to cdk4. The resulting cell line was named D1-KE and used in a tumor regression experiment, in which either D1 cells or D1-KE cells were injected into nude mice and tumors allowed to grow for 5 weeks before treatment. No significant difference in the growth of D1 and D1-KE xenografts was observed between weeks 1 and 5. At week 5, treatment was initiated. As observed previously (Fig. 4, C and D), fenretinide had a significant effect on the regression of the DF group (Fig. 5B) compared with DC (Fig. 5A; \( P/H_11005 0.001 \)) with a mean growth rate of 15% for the DC group and a growth rate of \( -5\% \) for the DF group (Fig. 5E). No significant difference in the growth rate was observed between DC xenografts (Fig. 5A) and D1-KEC (Fig. 5C; \( P/H_11021 0.92 \)). However, a highly...
significant reduction in growth rate was observed between D1-KEC (Fig. 5C) and D1-KEF (Fig. 5D) with a P of <0.0005 and a mean growth rate of 15% compared to −9%, respectively (Fig. 5E). Furthermore, statistical analysis between the DF group and the D1-KEF group suggested that tumor regression in the D1-KEF group was more pronounced than in the DF group (P = 0.033) as indicated by a mean growth rate of −9% and −1%, respectively. These results indicate that the cooperation between cyclin D1 and fenretinide treatment is independent of cyclin D1 binding to cdk4, and suggests that free cyclin D1 is responsible to generate a signal that lowers the threshold of fenretinide required to induce apoptosis.

**Fenretinide Promotes Tumor Regression of MCF-7 Xenograft.** MCF-7 cells overexpress cyclin D1 because of amplification of the cyclin D1 gene (21). As our data suggest that T47-D cells that express low levels of cyclin D1 are not sensitive to fenretinide treatment but that forcing the overexpression of cyclin D1 in these cells sensitizes them to fenretinide treatment, we next tested the effect of fenretinide on MCF-7 cells. Nude mice were injected with MCF-7 cells, and tumors were allowed to form for 3 weeks. As expected, no difference in the tumor growth rate was observed between mice assigned to the MCF-7 control group and the MCF-7-treated group between weeks 1 and 3. At week 3, treatment was initiated and tumor volume measured weekly. As observed for the D1 cells, fenretinide treatment led to the regression of MCF-7 xenografts (Fig. 6B) compared with the control group (Fig. 6A) as reflected by the mean growth rate of −2% and 14%, respectively. This result indicates that fenretinide promotes the regression of MCF-7 xenografts, a cell line that naturally overexpresses cyclin D1.

**DISCUSSION**

Cyclin D1 is overexpressed in 35% of breast cancers. Cyclin D1 was found recently to be essential for tumor formation in MMTV-erb-2 and MMTV-ras transgenic mice (10). This important finding indicated a significant role for cyclin D1 in breast cancer and immediately raised the possibility that drugs able to synergize with cyclin D1 may be very useful for the treatment of this significant subset of breast cancers. Fenretinide shows promising results for the chemoprevention of breast cancer (17). However, in this trial, patients were not selected for a specific molecular marker to predict response; therefore, it is possible that only a few patients would have expressed cyclin D1 in their tumors. Our results shown here indicate that overexpression of cyclin D1 sensitizes breast cancer cells to killing
by fenretinide. This finding is consistent with the observation that fenretinide delays the formation and reduces the size of tumors arising in MMTV-erb-2 transgenic mice, as these tumors overexpress cyclin D1 (9).

The mechanism of synergy between cyclin D1 overexpression and fenretinide remains unclear; however, the observation that the levels of exogenously expressed HA-cyclin D1 remain elevated in D1 cells after treatment with fenretinide (Fig. 1C) rules out the possibility that viability in the overexpressing cells is dependent on cyclin D1. Overexpression of cyclin D1 in cells in the absence of serum induces apoptosis, and this is thought to be the result of conflicting cell signaling (22). Although the precise mechanism underlying this effect remains undefined, the observation that overexpression of cyclin D1 sensitizes breast cancer cells to ATRA-induced apoptosis, a ligand that is normally a very poor inducer of apoptosis, is consistent with the ability of cyclin D1 to induce apoptosis under specific conditions. Because fenretinide, but not ATRA, can be used for the treatment of breast cancer, our data offers the first clinically relevant condition in which the effect of cyclin D1 on apoptosis is observed.

Furthermore, our results show that cyclin D1 binding to cdk4 is not required for the interaction between fenretinide and cyclin D1 to be observed. Therefore, free cyclin D1 appears to generate the signaling event that lowers the threshold of fenretinide-induced apoptosis and argues in favor of a model whereby the cooperation observed between cyclin D1 and fenretinide is linked to a negative role of cyclin D1 on cellular proliferation that is unrelated to its binding to cdk4. In agreement with such a model is our finding that cyclin D1 overexpression alone reduces tumor growth rate over an extended period of time in vivo (compare Fig. 4A with Fig. 4C). Furthermore, this observation may also offer a potential explanation to the surprising report of a better survival rate for breast cancer patients with elevated levels of cyclin D1 in their tumors (23).

We found that fenretinide treatment of xenografts overexpressing cyclin D1 is able to prevent tumor formation. These data are consistent with the indication of a benefit of fenretinide treatment in the prevention of breast cancer recurrence in premenopausal women (17). Whether the tumors of patients that did not recur after fenretinide treatment overexpressed cyclin D1 would have been very interesting to determine. However, the estimation of the statistical power of such study was low, and, therefore, could not reach statistical significance. Therefore, a new trial would be necessary to determine whether cyclin D1 overexpression predicts a positive response to fenretinide treatment. The importance of selecting patients for specific molecular marker is best illustrated by the fact that in a trial including 100 patients with a target response rate of 60% but in which only 10% of tumors express the appropriate molecular marker, the observed response rate will be only 6%. However, if patients are selected such that 100% of the patients express the molecular marker, the success rate of the trial is increased to 60%.

We also showed that fenretinide is able to promote the regression of xenografts overexpressing cyclin D1. Considering that fenretinide has been studied mostly in tumor prevention trials, this is potentially important as it suggests that fenretinide may be useful not only in preventing tumor recurrence, but also in the treatment of palpable tumors, at least potentially in the subpopulation of tumors that show elevated levels of cyclin D1. In conclusion, we have reported for the first time a positive interaction between fenretinide treatment and overexpression of cyclin D1. Fenretinide has the immense advantage of resulting in very few adverse side effects, to have a stable plasma con-

Fig. 6 Fenretinide treatment promotes regression of MCF-7 xenografts. A, at week 0, 10 nude mice were injected with MCF-7 cells and at week 3 received mock treatment (A) or fenretinide (B). Tumor growth was determined over a period of 8 weeks as described in "Materials and Methods." E, tumor growth rate for each group of mice. N indicates the number of mice per group remaining after 8 weeks. The mean and the 95% CI are shown below each group.
centration and to concentrate in the breast. Furthermore, overexpression of cyclin D1 has been proposed to promote resistance to Tamoxifen (24), an observation that emphasizes the importance of identifying therapeutic alternatives for the treatment of cyclin D1-overexpressing tumors. Therefore, considering the clinical advantages that fenretinide offers and the high percentage of breast cancers that show abnormal elevation in cyclin D1 levels, the interaction between cyclin D1 and fenretinide represents a promising step toward the design of a nontoxic and targeted therapy for breast cancer treatment.

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