Targeting the EWSR1-FLI1 Oncogene-Induced Protein Kinase PKC-β Abolishes Ewing Sarcoma Growth

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Abstract

Ewing sarcoma is a rare but aggressive disease most common in young adults. This cancer is driven by a unique chimeric fusion oncogene but targeted strategies have been elusive. Here we report the identification of the protein kinase PKC-β (PRKCB) as a disease-specific druggable target for treatment of Ewing sarcoma. We found that transcriptional activation of PRKCB was directly regulated by the chimeric fusion oncogene EWSR1-FLI1 that drives this cancer. PRKCB phosphorylated histone H3T6 to permit global maintenance of H3K4 trimethylation at a variety of gene promoters. PRKCB loss induced apoptosis in vitro and prevented tumor growth in vivo. Gene expression profiling revealed a strong overlap between genes modulated by EWSR1-FLI1 and PRKCB in regulating crucial signaling pathways. Taken together, our findings offer a preclinical proof-of-concept for PRKCB as a promising therapeutic target in Ewing sarcoma. Cancer Res. 72(17): 1–10. ©2012 AACR.

Introduction

Ewing sarcoma is the second most frequent childhood bone tumor. Clinically, Ewing sarcoma is a highly metastatic tumor with around 25% of patients presenting metastasis at the time of diagnosis. Although great advances have been made in the treatment of local disease, therapies used for advanced stages of the disease are still disappointing, the 5-year overall survival still being critically low. Thus the discovery of novel druggable targets allowing targeted therapies is mandatory.

Since the discovery and characterization of the causal translocation event (1, 2), researches were driven by the quest for targets of the chimeric EWSR1-FLI1 transcription factor, the main oncogenic event in more than 85% of Ewing sarcoma. Most of the studies aiming at identifying important molecules involved in Ewing sarcoma oncogenesis were based either on EWSR1-FLI1 inhibition in Ewing sarcoma cells or EWSR1-FLI1 expression in non-Ewing cell lines. Thus, many different studies, including ours, identified genes involved in EWSR1-FLI1–dependent cell survival, such as IGF-1R (3) and IGFBP (4, 5), PPP1R1A (6), MK-STYX/STYXL1 (7), NR0B1 (8, 9), NX2-2 (10), GSTM4 (11), and SOX2 molecules (12). Proposed as a promising targeted therapy, IGF signaling inhibition has been extensively investigated in the context of Ewing sarcoma, and several clinical trials have been conducted. Although well tolerated, several reports of phase II studies suggested that only about one patient of 4 would benefit from IGF-1R monoclonal antibodies as single therapy (13). On the other hand, some studies analyzed primary tumors to find specific gene signature of Ewing sarcoma (6, 14–16).

Aiming at finding genes that are specifically found in primary Ewing tumors compared with other tumors types and modulated by EWSR1-FLI1, we identified the protein kinase Cβ (PRKCB). In the context of Ewing sarcoma, we show here that PRKCB is crucial for cell survival in vitro and tumor development in vivo. We show that inhibition of PRKCB induces apoptosis and stimulates TNF and NF-κB signaling and that concomitant inhibition of both PRKCA and PRKCB, by pharmacologic inhibition or RNA silencing, is required to decrease histone H3 lysine 4 methylation levels.

Materials and Methods

Microarrays and statistical analysis

Ewing tumors samples (GSE34620), together with samples of other pediatric or bone tumors including 32 small round cell desmoplastic tumors, 52 medulloblastomas (GSE12992), 64 neuroblastomas (GSE12460), 122 rhabdomyosarcoma (17), 27 osteosarcomas (GSE14827), and 34 synovial sarcomas (GSE20196), were used for this analysis. All microarray data...
were simultaneously normalized using the germa package version 2.18.1 in R 2.10.1 environment (18). BGA analyses were completed using mad4 R package (19). For siRNA inhibition microarrays, A673 cells were transfected by the indicated siRNA and harvested 72 hours posttransfection. Total RNAs were extracted using TRIzol Reagent and processed on Affymetrix HuGene L.1STv1 GeneChip microarrays (GEO accession number GSE3892). RNA normalization was carried out using Brainarray Entrez gene CDF v14.1 (20) in R environment. Group comparisons were done using Welch 2 sample t test statistic and DAVID gene ontology analyses (21) were carried out online (http://david.abcc.ncifcrf.gov/home.jsp). Gene set enrichment analysis (GSEA; ref. 22) was done by comparing siPRKCB and siControl samples using a log ratio of classes metric.

**In vivo mice experiments**

For A673-shPRKCB463_B5M3 xenograft tumors in severe combined immunodeficient (SCID) mice, 20 millions cells resuspended in 200 μL of PBS were injected subcutaneously in the flank of 5-week-old SCID female mice (Charles River laboratories). Doxycycline was added to the drinking water at 2 mg/mL, together with 5% sucrose. Tumor volume was determined by caliper measurements every 2 to 3 days until volume reached 1,500 mm³. For the A673 xenograft Ewing sarcoma model in nude mice, 20 millions cells were injected next to the tibia of 4-week-old male athymic mice (Janvier, L’Arbresles, France) under anesthesia [inhalation of a combination of isoflurane/air (1.5%, 1 L/min) and buprenorphine (0.05 mg/kg; Temgesic, Schering-Plough)]. Once tumors were measurable, mice were randomly assigned into treatment groups receiving either oral gavage with enzastaurin suspended in olive oil (100 mg/kg, twice daily; flurane/air (1.5%, 1 L/min) and buprenorphine (0.05 mg/kg; Temgesic, Schering-Plough)]. All control spots (normal tissues or a high-grade myxoid liposarcoma -SAB- cell line) were carried out by CO2 inhalation. All control spots (normal tissues or a high-grade myxoid liposarcoma —SAB—) were positive for PRKCB2 stainings. In addition, mean value of PRKCB expression in Ewing tumors is 13 fold superior to mean value of the closest group (neuroblastomas). Although 2 isoforms of PRKCB (PRKCB1 and PRKCB2) distinguished by their differentially spliced last exon have been described (hereafter, we will indicate PRKCB when referring to both isoforms), PRKCB2 is the predominant isoform in Ewing sarcoma as compared with other non-Ewing cancer cell lines (Fig. 1C and Supplementary Fig. S1). We confirmed the massive expression of PRKCB at the protein level on an Ewing sarcomas tissue microarray, in which all 11 Ewing sarcoma samples were positive for PRKCB2 staining. All control spots (normal tissues or a high-grade myxofibrosarcoma), together with 2 other pediatric tumors (one desmoplastic small round cell tumor and one synovial sarcoma), tested negative (Fig. 1D). Strong PRKCB overexpression thus seems to be highly specific of Ewing tumors.

**PRKCB expression is directly dependent on EWSR1-FLI1**

Inhibition of EWSR1-FLI1 using siRNA in A673, EW24, and SK-N-MC Ewing cell lines led to fast and proportional reduction of PRKCB expression at both RNA and protein levels (Fig. 2A and B and Supplementary Fig. S2B), indicating that PRKCB expression in Ewing sarcoma cells is EWSR1-FLI1 dependent. To assess the direct role of EWSR1-FLI1 in PRKCB expression, luciferase reporter experiments with PRKCB promoter (from −1,147 to +22 according to transcription start site) were conducted. As shown in Fig. 2C, ectopic expression of EWSR1-FL1 in HeLa cell line strongly increased luciferase activity. In addition, in shA673-1C cell line, which allows a doxycycline-mediated inhibition of EWSR1-FL1 (31), PRKCB promoter activity was significantly decreased upon EWSR1-FLI1 inhibition (Fig. 2D). Finally, ChIP by 2 different EWSR1-FL1 antibodies conducted in A673 cells indicated the presence of EWSR1-FL1 within the PRKCB promoter in vivo (Fig. 2E). These different results clearly indicated that EWSR1-FL1 is directly responsible for the high expression of PRKCB in Ewing cell lines.

**PRKCB is an active kinase in Ewing sarcoma and is responsible for histone H3T16 phosphorylation**

In a recent publication on prostate cancer cells (32), PRKCB was shown to phosphorylate histone H3 on its threonine 6

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**Results**

**PRKCB is strongly overexpressed in Ewing tumors**

To find specific genes expressed in Ewing sarcoma, a supervised between-group analysis (25) was carried out comparing expression profiles of 39 Ewing tumors samples with those of other pediatric tumors, including 32 small round cell desmoplastic tumors, 52 medulloblastomas (26), 64 neuroblastomas (27), and 122 rhabdomyosarcoma (ref. 17; Fig. 1A). Among the top 100 probe sets found to be specific for Ewing sarcoma (see Supplementary Table S1), several represented genes that were previously identified as important for Ewing tumors such as NXK2-2 (10), NR0B1 (8, 9), LIP1 (28), DKK2 (29), or XR (30), and most of them were previously identified as EWSR1-FL1-dependent genes (4, 15, 31). Among these genes, we focused our attention on the PRKCB for which 5 probe sets (Fig. 1A) were significantly overexpressed in Ewing tumors, as compared with other pediatric or bone tumors (Fig. 1B). Indeed, mean value of PRKCB expression in Ewing tumors is 13 fold superior to mean value of the closest group (neuroblastomas). Although 2 isoforms of PRKCB (PRKCB1 and PRKCB2) distinguished by their differentially spliced last exon have been described (hereafter, we will indicate PRKCB when referring to both isoforms), PRKCB2 is the predominant isoform in Ewing sarcoma as compared with other non-Ewing cancer cell lines (Fig. 1C and Supplementary Fig. S1). We confirmed the massive expression of PRKCB at the protein level on an Ewing sarcomas tissue microarray, in which all 11 Ewing sarcoma samples were positive for PRKCB2 staining. All control spots (normal tissues or a high-grade myxofibrosarcoma), together with 2 other pediatric tumors (one desmoplastic small round cell tumor and one synovial sarcoma), tested negative (Fig. 1D). Strong PRKCB overexpression thus seems to be highly specific of Ewing tumors.
(H3T6ph), leading to a decreased ability for the cell to remove di/tri-methyl tags on lysine 4 of H3 (H3K4me2/me3). We therefore investigated in Ewing sarcoma whether PRKCB overexpression correlated with high H3T6 phosphorylation—and consequently H3K4 methylation—levels and whether this effect was related to EWSR1-FLI1 direct DNA binding. Promoter regions of 13 known EWSR1-FLI1—modulated genes (7 induced: AURKB, CAV1, CCND1, EZH2, NKX2.2, NR0B1, PRKCB, ROCK1, SNF8, TERT, ROCK2, and SNF8) were selected according to their homology with PRKCB. For instance, both PRKCB and PRKCA are at least on most of tested promoters.

Altogether, we showed here that PRKCB is responsible, together with PRKCA, for maintaining steady-state H3T6ph and H3K4me3 levels, at least on most of tested promoters.

Roles of PRKC in Ewing Sarcoma Biology

Figure 1. PKCB expression in Ewing tumors. A. between group analysis of expression profiles from 5 pediatric tumor types. Samples (colored circles) are separated along axes together with probe sets (light gray dots) responsible for their separation. Position of probe sets corresponding to NR0B1, NKX2-2, and PRKCB are emphasized (colored stars). B. box plot of PRKCB expression (log2 intensity of Affymetrix hu133plus2 probe set 227817_at) among several pediatric or bone tumors. ES, Ewing sarcomas; MB, medulloblastomas; NB, neuroblastomas; DSRCT, desmoplastic small round cell tumors; OS, osteosarcomas; RMS, rhabdomyosarcomas; SV, synovial sarcomas. C, expression of PRKCB1 and PRKCB2 in 3 Ewing cell lines (A673, EW24, and SK-N-MC) as compared with non-Ewing cell lines (HeLa, Jurkat, and SJNB1). β-Actin was used as loading control. D, immunohistochemistry for PRKCB2 expression pro in vitro (32). In addition, although enzastaurin is a specific PRKCB inhibitor (IC50, 6 nmol/L), it also clearly displays inhibitory effect against PRKCA (IC50, 39 nmol/L; ref. 33). Consequently, as PRKCA is expressed in Ewing cell lines (Supplementary Fig. S1 and ref. 34), we hypothesized that either PRKCA or PRKCB could be sufficient to maintain H3T6ph and H3K4me3 levels. ChIP experiments were then carried out in A673 cells transfected either with siPRKCB or siPRKCA alone or with both siRNAs. As postulated, only the combinatorial inhibition of PRKCA and PRKCB led to a drastic reduction of H3T6 phosphorylation and H3K4 trimethylation levels (Fig. 3E and F), whereas inhibition of PRKCA alone did not decrease H3K4me3 (Supplementary Fig. S3). Altogether, we showed here that PRKCB is responsible, together with PRKCA, for maintaining steady-state H3T6ph and H3K4me3 levels, at least on most of tested promoters.

To our surprise, inhibition of PRKCB did not significantly affect H3T6ph or H3K4me3 levels (Fig. 3C and D). To account for this result, we hypothesized that other members of classical PKCs subfamily may complement PRKCB deficiency. PRKCA is of particular interest as it shares high structural and functional homology with PRKCB. For instance, both PRKCB and PRKCA can phosphorylate H3T6 in vitro (32). In addition, although enzastaurin is a specific PRKCB inhibitor (IC50, 6 nmol/L), it also clearly displays inhibitory effect against PRKCA (IC50, 39 nmol/L; ref. 33). Consequently, as PRKCA is also expressed in Ewing cell lines (Supplementary Fig. S1 and ref. 34), we hypothesized that either PRKCA or PRKCB could be sufficient to maintain H3T6ph and H3K4me3 levels. ChIP experiments were then carried out in A673 cells transfected either with siPRKCB or siPRKCA alone or with both siRNAs. As postulated, only the combinatorial inhibition of PRKCA and PRKCB led to a drastic reduction of H3T6 phosphorylation and H3K4 trimethylation levels (Fig. 3E and F), whereas inhibition of PRKCA alone did not decrease H3K4me3 (Supplementary Fig. S3). Altogether, we showed here that PRKCB is responsible, together with PRKCA, for maintaining steady-state H3T6ph and H3K4me3 levels, at least on most of tested promoters.
PRKCB is crucial for Ewing cell survival in vitro

Knowing that PRKCB was active in Ewing sarcoma cells, we next investigated whether PRKCB expression was necessary for cell survival. For this purpose, A673, SK-N-MC, and TC71 cell lines were infected with lentiviral particles containing short hairpin RNAs (shRNA) against PRKCB (sh463 and sh847) that elicited strong PRKCB inhibition (5). As several compounds have been previously reported to specifically inhibit PKCB activity, we investigated whether pharmacologic PRKCB inhibition was also effective in Ewing sarcoma. We choose to investigate enzastaurin (33) as this molecule has been fostered to phase II/III clinical trials for several diseases. Growing Ewing sarcoma A673, SK-N-MC, and TC71 cell lines with increasing amount of enzastaurin led to a dramatic reduction of cell viability with an IC₅₀ around 5 μmol/L. Altogether, these results indicated that in vitro inhibition of PRKCB strongly induces cell death via apoptotic pathways.
PRKCB inhibition induces tumor regression in vivo

We next wondered whether we could recapitulate in vivo the tremendous apoptotic effect of PRKCB inhibition observed in vitro. For this purpose, we generated the shA673-B5M3 Ewing cell line that expresses the shRNA463 directed against PRKCB under the control of a doxycycline-inducible promoter (Supplementary Fig. S5). Xenograft tumors were then raised into SCID mice by subcutaneously implanting shA673-B5M3 cells or control cells (shA673-pTER). Remarkably, shA673-B5M3 xenograft tumor growth was completely abolished when mice were treated with doxycycline as soon as 4 days after cells inoculation (Fig. 5A). Moreover, shRNA-mediated PRKCB inhibition reduced tumor size very significantly when mice were doxycycline-treated once tumors were detectable (Fig. 5B). Finally, A673 cells were injected at the bone periphery, into mouse tibial muscle, to mimic the human disease. In these mice, treatment with enzastaurin led to a significant reduction of tumor growth (Fig. 5C). Overall, these 3 in vivo experiments clearly showed that blocking PRKCB prevents Ewing sarcoma tumor engraftment, reduces tumor growth, and promotes tumor reduction.

Roles of PRKC in Ewing sarcoma

We showed that pharmacologic inhibition of PRKC led to a decreased histone phosphorylation and that PRKB inhibition led to apoptosis, but it is unlikely that these 2 mechanisms are

Figure 3. Synergistic effect of PRKCA and PRKCB for the prevention of histone demethylation in Ewing sarcomas assessed by chromatin immunoprecipitation. Quantitative PCR targeting trimethylated promoter/enhancer regions of known EWSR1-FLI1–modulated genes (7 induced and 6 repressed) as well as 6 EWSR1-FLI1–unmodulated genes are shown. qPCR values for each gene/ChIP conditions were normalized to their respective input. Means and SDs of 2 replicates are shown. A, C, and E, ChIP with an antibody against histone H3T6 phosphorylation (H3T6ph). B, D, and F, ChIP with an antibody against histone H3K4 trimethylation (H3K4me3). A and B, A673 cells treated for 72 hours with 6 μM of enzastaurin or with equivalent amount of DMSO. C and D, A673 cell transfected with siRNA targeting PRKCB (siPRKCB) or with nontargeting negative siRNA control (siCT). E and F, A673 cell transfected with both siRNA targeting PRKCB and PRKCA (siPRKCA+siPRKCB) or with nontargeting negative siRNA control (siCT).
related as PRKCB inhibition had no effect on histone modification. Thus, to trying to decipher the molecular roles of PRKCB in Ewing sarcoma, we carried out expression profiling on A673 Ewing cell lines treated by specific siRNA against either PRKCB or EWSR1-FLI1 and with a nontargeting siRNA as control. Supervised differential analyses between samples treated by either specific siRNA and samples treated with the control siRNA were done, and the lists of genes modulated in the different conditions were compared with each other (Fig. 6A and Supplementary Table S2). As expected, we found downregulation of many histones/chromatin/chromosome organization gene categories, reinforcing the possible link between EWSR1-FLI1 and chromatin remodeling through PRKCB (Supplementary Table S3). More strikingly, we observed an extremely significant overlap while comparing EWSR1-FLI1- and PRKCB-modulated genes. Indeed, more than half of the genes modulated upon PRKCB inhibition were also modulated during EWSR1-FLI1 inhibition (Fig. 6A). Looking at the gene ontology or pathways modulated by either siRNA inhibition (see Supplementary Table S3), cell death was significantly upregulated, which was consistent with the observed phenotype. Indeed, during PRKCB inhibition, caspase-3, caspase-8, and TNFRSF12A (Tweak-receptor) were strongly upregulated and are known to induce apoptosis in some tumors (35). Furthermore, GSEAs showed that PRKCB inhibition led to a significant increase of TNF and NF-kB pathways, also possibly leading to apoptosis (Fig. 6B). Altogether, our results showed that PRKCB possesses at least 2 different functions in Ewing sarcoma, the first being its involvement in crucial signaling pathways leading to rapid cell death when impaired, the second being its responsibility for histone H3T6 phosphorylation, leading to long-term impact on transcriptional activity.
Interestingly enough, we show that inhibition of PRKCB focused our attention on histone H3T6 phosphorylation. Interor BimEL (zum Buschenfelde and colleagues; 2010), but we the literature such as AKT (Graff and colleagues; 2005) and Bcl2. Numerous potential PRKCB substrates have been described in increased activity, we investigated whether PRKCB was active.

sarcoma. Converge to increase expression level of PRKCB in Ewing through, respectively, EWSR1-FLI1 and VEGF signaling may controls (37). Thus, direct and indirect activation of promoters experiments. Noticeably, VEGF was recently shown to stimulate PRKCB expression in chronic lymphocytic leukemia cells (36). It is notable that Ewing sarcoma patients display increased serum VEGF levels as compared with healthy controls (37). Thus, direct and indirect activation of PRKCB through, respectively, EWSR1-FLI1 and VEGF signaling may converge to increase expression level of PRKCB in Ewing sarcoma.

As overexpression is not necessarily correlated with increased activity, we investigated whether PRKCB was active. Numerous potential PRKCB substrates have been described in the literature such as AKT (Graff and colleagues; 2005) and Bcl2 or BimEL (zum Buschenfelde and colleagues; 2010), but we focused our attention on histone H3T6 phosphorylation. Interestingly enough, we show that inhibition of PRKCB is not sufficient to modify H3T6 phosphorylation pattern on a selected set of genes. Eventually, we show here that either PRKCB or PRKCA expression is sufficient to prevent histone H3K4 demethylation in Ewing sarcoma as PRKCB and PRKCA should be inhibited together to see an effect on histone H3T6 phosphorylation, therefore indicating a redundant function. Nevertheless, we cannot rule out a peculiar effect of PRKCB on specific promoters that were not tested here. In prostate cancer cells, upon treatment with R1881—a synthetic agonist of the androgen receptor—Metzger and colleagues have shown that PRKCB was able to phosphorylate H3T6 only in the promotor region of androgen-responsive genes. A similar mechanism could be involved in Ewing sarcoma. Despite the lack of a direct interaction observation, we could hypothesize a process in which PRKCB interacts, directly or not, with EWSR1-FLI1 on a restricted set of promoter, leading to either transcriptional activation or repression of the corresponding genes.

Expression profiling experiments showed a strong overlap between genes modulated upon EWSR1-FLI1 knockdown and those modulated by PRKCB inhibition. To account for this result, 2 hypotheses could be made (i) PRKCB may directly phosphorylate EWSR1-FLI1, therefore, affecting its activity or stability. Nevertheless, preliminary experiments did not allow us to detect PRKCB-driven EWSR1-FLI1 phosphorylation, but

![Figure 5. Crucial importance of PRKCB for in vivo Ewing cell survival.](image-url)

Discussion

We identified PRKCB as a major Ewing-specific protein, in which overexpression is directly linked to EWSR1-FLI1. Indeed, bioinformatics approaches led to the identification of PRKCB among the most overexpressed gene in Ewing sarcomas, which could be confirmed using quantitative reverse transcriptase PCR (RT-PCR) and in situ using immunohistochemistry on tissue microarrays. Moreover, using siRNA inhibition experiments, we show here a direct regulation of PRKCB expression by EWSR1-FLI1 that is validated by ChIP and luciferase reporter promoter experiments. Noticeably, VEGF was recently shown to stimulate PRKCB expression in chronic lymphocytic leukemia cells (36). It is notable that Ewing sarcoma patients display increased serum VEGF levels as compared with healthy controls (37). Thus, direct and indirect activation of PRKCB through, respectively, EWSR1-FLI1 and VEGF signaling may converge to increase expression level of PRKCB in Ewing sarcoma.

![Figure A](image-url)

![Figure B](image-url)

![Figure C](image-url)
more sophisticated experiments are required, and enzastaurin inhibition of PRKCB did not have an effect on EWSR1-FLI1 expression level (Supplementary Fig. S6). (ii) PRKCB may induce chromatin modification of many EWSR1-FLI1 target genes. However, we show that both PRKCA and PRKCB are expressed in Ewing sarcoma and that they share redundant functions on IJ16 phosphorylation, leading to a more complex regulation of chromatin status as originally thought.

We also show here that PRKCB is a major therapeutic target in Ewing sarcoma. Indeed, inhibition of PRKCB either by small molecules inhibitors or by siRNA induces apoptosis. Therefore, PRKCB inhibition in Ewing cell lines has rather a cytotoxic than a cytostatic effect that is of strong interest for therapeutic use. Moreover, we found an average enzastaurin IC50 of 5 μmol/L, which is in the concentration range of sensitivity found in most of the data published for this drug. As an example, in 2 recent publications on lung cancer and colon cancer, cells were considered sensitive when IC50 was 10 μmol/L or less (39), respectively. Interestingly, lymphomas and leukemias, which are sensitive to enzastaurin (37), also express elevated levels of PRKCB (see Supplementary Fig. S1A). Tested on a panel of different kinases, enzastaurin was shown to be a specific inhibitor of the protein kinase C family, especially PRKCB and PRKCA (33). We thus investigated PRKCA characteristics in Ewing sarcoma (Supplementary Fig. S2) and found that (i) PRKCA seemed less expressed in Ewing sarcoma than in other tumor types. (ii) Unlike PRKCB, PRKCA expression was increased upon EWSR1-FLI1 inhibition, indicating a repression by the fusion protein. (iii) This repression was most probably through indirect mechanisms as no communoprecipitation of the promoter could be found in our EWSR1-FLI1 ChIP experiments or in previous ChIP-seq publications (23, 40). Moreover, this expression modulation was observed only 72 hours after EWSR1-FLI1 inhibition, whereas PRKCB modulation is seen as early as 24 hours. (iv) Most importantly, upon inhibition of PRKCA, we observed a slight inhibition of proliferation. Nevertheless, in Ewing sarcoma PRKCA was previously reported to regulate resistance to drugs such as cisplatin and doxorubicin, trough caveolin-1 (CAV1)-dependent mechanism (34). This last work highlights a significant role of the alpha isoform. Thus, the simultaneous inhibitory effect of enzastaurin on PRKCB and PRKCA may be considered as a therapeutic advantage.

However, specific shRNA inhibition of PRKCB is sufficient to impair tumorigenesis of Ewing cells in vivo. To our knowledge and apart from EWSR1-FLI1 inhibition (41), this is the first demonstration of a single gene knockdown leading to tumor shrinkage in Ewing xenograft models, opening interesting therapeutic opportunities based on PRKCB extinction. Usage of specific drugs such as enzastaurin also proved its efficacy for inhibiting tumor growth in vivo but to a lesser extent than with RNA inhibition. Accounting for this result, enzastaurin accumulation in mice adipose tissue was observed in these experiments (data not shown), leading to its reduced bioavailability at tumor sites.

Relative to other preclinical data using enzastaurin, our results are encouraging as we saw a rather robust delay in growth and a quick response to the drug. As a single therapeutic molecule, PRKCB inhibitors proved to be rather inefficient in delaying tumor growth in preclinical studies on lung (42) or pancreatic (43) cancers, whereas on multiple myeloma (44) or transitional cell carcinoma (45), responses were equivalent to our observations. In Waldenström macroglobulinemia (46) and glioblastomas (33), the PRKC inhibitor proved to be clearly efficient. Nevertheless, significant differences observed in these last studies were seen 2 to 5 weeks after the treatment starting point whereas in our experiments, we saw a significant effect as soon as 5 days after first drug administration. This indicates a high responsiveness of Ewing sarcoma cells to this class of drugs. An orthotopic Ewing mouse model presenting a slower growth rate could thus be advantageous to study PRKC inhibition. In addition, it may be interesting to see the effect of...
PRKCB inhibitors on metastases growth, but this will require reliable Ewing metastatic models.

In this study, we clearly showed the antiapoptotic properties of PRKCB in the context of Ewing sarcoma, as its inhibition is sufficient to robustly trigger apoptosis. To account for this effect, microarray data indicate a significant increase of TNF superfamily death receptor upon PRKCB silencing. Previous report on Jurkat or HL-60 human leukemia cell showed that inhibition of PRKCB sensitized tumor cells to TNF-α-related apoptosis via ligand-induced death receptor (47). Similar mechanisms may occur in Ewing sarcoma. Indeed, activation of NF-kB pathway by TNF-α in preliminary experiments indicated that TNF-α markedly increased ezastaurin-mediated death of A673 cells (see Supplementary Fig. S7). However, further experiments will be required to get a comprehensive picture of PRKCB involvement in these processes.

PRKCB, as a kinase, possesses an enzymatic activity that can be directly targeted and we show here that blocking PRKCB in Ewing sarcoma is a new promising therapeutic approach.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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