ACS OMEGA

Open Access

http://pubs.acs.org/journal/acsodf

Article

PRIMA-1^{MET} Does Not Restore Vitamin D Sensitivity to MDA-MB-231 and MDA-MB-468 Triple-Negative Breast Cancer Cells

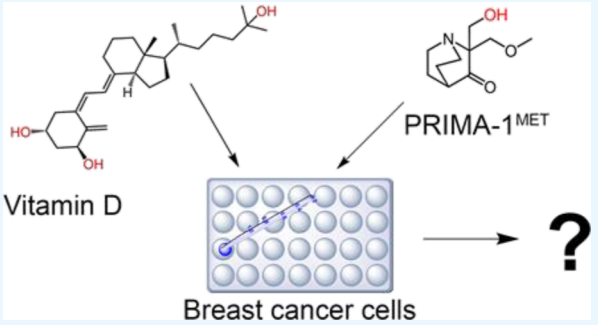
Shadi N. Kotob and Jessica L. Kelts*

Cite This: *ACS Omega* 2023, 8, 30500−30507

Read Online

ACCESS | Metrics & More | Article Recommendations

ABSTRACT: Vitamin D is a steroid hormone that causes growth suppression in cultured cells. We had previously discovered that the triple-negative breast cancer cell lines MDA-MB-231 and MDA-MB-468 did not have growth suppression with vitamin D, while MCF-7 did. MCF-7 cells are not triple-negative and have wild-type p53. Both MDA-MB-231 and MDA-MB-468 have mutations in p53 and these mutations were a possible explanation for the lack of growth suppression with vitamin D. Our hypothesis was that reactivation of p53 in the triple-negative cell lines would cause them to become sensitive to vitamin D. We chose to use the small molecule PRIMA-1^{MET} to reactivate p53 as it has been previously shown to restore function to the p53 mutants present in MB-231 and MB-468. We then measured the ability of vitamin D and its analogues calcipotriol and EB1089 to suppress growth in the presence of PRIMA-1^{MET}. Here, we show that while PRIMA-1^{MET} can kill the breast cancer cells investigated in this study, it does not restore their sensitivity to vitamin D or its analogues.



INTRODUCTION

Breast cancer is the second largest cause of death for women in the United States.[1] Among the different types of breast cancer, triple-negative breast cancer (TNBC) represents 15−20% of cases and can have a mortality rate of up to 40% for late-stage disease.[2] TNBC occurs when the cancer cells do not express estrogen receptors, progesterone receptors, or the human epidermal growth factor receptor 2 (HER2). Lack of these receptors prevents the use of common chemotherapeutics that would target them, such as tamoxifen, an anti-estrogen, or Herceptin, which targets HER2 overexpression.[3,4]

The effects of activated 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$, or calcitriol) and its circulating precursor 25-hydroxyvitamin D_3 (25-OHD_3) on breast cancer are not conclusively settled.[5] However, some recent studies have linked either increased circulating vitamin D_3 levels, increased vitamin D_3 intake, or sun exposure to decreased cancer incidence or survival.[6−8] Presence of vitamin D receptor (VDR) has been linked to longer survival in TNBC[9] and in breast cancer overall.[10] 1,25$(OH)_2D_3$ has also been shown to cause VDR mediated suppression of genes related to invasion and metastasis in TNBC.[11] These studies indicate a role for vitamin D in prevention and treatment of various cancers. However, a 2021 review on the mechanisms of vitamin D in breast cancer notes that the lack of clarity on the determinants of vitamin D sensitivity is preventing the formation of clinical protocols or official recommendations about vitamin D.[5]

We had previously established that unlike with many other cancer cell lines, 1,25$(OH)_2D_3$ caused either no change or increased cell viability with the TNBC cell lines MDA-MB-157, MDA-MB-231, and MDA-MB-468.[12] The cell viability of MCF-7 cells decreased with increased 1,25$(OH)_2D_3$, as would be expected. One of the hypotheses for this phenomenon was the mutated p53 expressed for the three TNBC cell lines, while MCF-7 expresses wild-type p53.[13] Our studies identified one cell line that was both triple-negative and had wild-type p53; however, that cell line (DU4475) also had a slight increase and then decrease in cell viability with increasing 1,25$(OH)_2D_3$.[14] We were not satisfied that results with this one cell line were enough to outright dismiss the hypothesis about mutant p53 causing abnormal 1,25$(OH)_2D_3$ response.

To further test the hypothesis, we sought to reactivate p53 in these cell lines. We turned to the compound PRIMA-1^{MET} (APR-246, eprenetapopt), which is a small molecule activator of mutant p53. PRIMA-1^{MET} and its precursor molecule PRIMA-1 are both converted to methylene quinuclidinone, which binds to the core domain of mutant p53, allowing it to

Received: May 26, 2023
Accepted: July 17, 2023
Published: August 8, 2023

function.[15,16] There are numerous studies showing restoration of p53 function using either PRIMA-1 or PRIMA-1^{MET}.[16−18] MB-231 cells express p53 bearing an R280K mutation and MB-468 cells express p53 bearing an R273H mutation.[19,20] p53 bearing both mutations has been shown to be activated by PRIMA-1^{MET}.[17,21] Clinical interest in these p53 reactivating small molecules yielded several clinical trials in various cancer types that can be found on ClinicalTrials.gov.

The availability of this small molecule that has been shown to reactivate mutant p53 in our TNBC cell lines enables us to probe whether mutant p53 is responsible for the aberrant response to 1,25$(OH)_2D_3$ in MB-231 and MB-468. In this study, we aim to test the hypothesis that PRIMA-1^{MET} will restore 1,25$(OH)_2D_3$ sensitivity to MB-231 and MB-468 is correct.

METHODS

Materials. Materials were purchased from Fisher Scientific (Waltham, MA, USA) unless otherwise noted. Cell lines (MCF-7, MDA-MB-231, and MDA-MB-468) were obtained from ATCC (Manassas, VA, USA). PRIMA-1^{MET}, 1,25-$(OH)_2D_3$, and calcipotriol were purchased from Cayman Chemical (Ann Arbor, MI, USA), and EB1089 was purchased from Tocris Bioscience (Minneapolis, MN, USA). CellTiter-Glo was obtained from Promega (Madison, WI, USA).

Cell Culture. Cells were maintained in complete culture medium: DMEM (MDA-MB-468 and MCF-7) or MEM (MDA-MB-231), 10% fetal bovine serum (FBS, Gibco), 1× Glutamax, 1× antibiotic/antimycotic (LifeTechnologies, Carlsbad, CA, USA), and 1 mM sodium pyruvate. MCF-7 cells also had 10 μg/mL insulin added to the media. Cells were passaged twice per week using 0.25% Trypsin−EDTA. Stripped-serum media was used for all experiments. These media were analogous to the complete media formulations, but without sodium pyruvate and with charcoal stripped FBS (Gibco) in place of the normal FBS.

Cell Viability Experiments. Cells were plated in white cell culture treated 96-well plates at a density of 1000 cells in 80 μL. Empty wells contained phosphate buffered saline (PBS). Treatment compounds were added the following day, bringing the volume to 100 μL. Three days later, 100 μL CellTiter-Glo was added and luminescence was recorded using a GloMax Multi plate reader (Promega) after 20−30 min at room temperature. Vehicle was constant across each plate and ranged from 0.048−0.066% ethanol. Additionally, calcipotriol and EB1089 plates contained 0.001−0.005% DMSO. Graphs were made using GraphPad Prism 9.5.1. p-values were determined using Prism to perform two-way ANOVA with Dunnet post hoc test. For each experiment, we performed four technical replicates for each of three biological replicates.

Cell Lysates and Immunoblots. The day before treatment, 1 million cells were plated in T-25 flasks in stripped-serum media. Cells were then treated with PRIMA-1^{MET} and/or ethanol (all flasks 0.08% ethanol). After 23−25 h, cells in the media were harvested by centrifugation, and cells attached to the flask were harvested by trypsinization. The cells were combined after resuspension in complete media and washed once with ice-cold PBS. The pellet was then resuspended in 200 μL of ice-cold RIPA buffer (Pierce RIPA buffer plus 1× Halt protease and phosphatase inhibitors, 1× Halt EDTA, and 1× Pierce Universal Nuclease). The mixtures were rocked in RIPA buffer for at least 30 min at 4 °C. Cell debris was pelleted by centrifugation for 15 min at 14,000 × g at 4 °C. The supernatant was moved to a fresh microfuge tube, and protein concentrations were determined using the BCA assay (Pierce) in microplate format.

Gel samples were prepared using fluorescent compatible 4× sample buffer and 10× reducing agent (Invitrogen). Proteins were separated on 4−12% Bis-Tris Plus gels using MES-SDS buffer (Invitrogen). Proteins were transferred to PVDF membranes using the iBlot2 system (Invitrogen). Total protein was then labeled on the membrane using No Stain Protein Labeling reagent (Invitrogen) as per manufacturer's instructions. 5% milk in PBS + Tween-20 was used as a blocking buffer for 1 h at room temperature. The primary antibody (p53 monoclonal, Invitrogen DO-1) was used at 1:1000 in blocking buffer overnight at 4 °C. The secondary antibody (goat anti-mouse HRP, Thermo Scientific) was used at 1:200,000 in blocking buffer for 1 h at room temperature. Bands were detected using Supersignal West Femto Substrate (Thermo Scientific). Chemiluminescent p53 bands and fluorescent total protein staining were imaged on an iBright FL 1500 imaging system, and p53 band intensity was normalized to total protein content of each lane.

RESULTS AND DISCUSSION

In addition to testing 1,25$(OH)_2D_3$ in this study, we also included the vitamin D analogues calcipotriol and EB1089. Achieving high blood levels of endogenous vitamin D_3 in vivo leads to hypercalcemia, which has its own complications. This led to the development of analogues of vitamin D that are less calcemic. Calcipotriol was shown to be much less calcemic in rats as compared to 1,25$(OH)_2D_3$.[22,23] EB1089 was also found to be less calcemic at effective doses.[24,25] Therefore, the inclusion of these vitamin D analogues in our research allows our results to have more potential clinical application.

As seen in Figure 1A, 1,25$(OH)_2D_3$, calcipotriol, and EB1089 inhibit MCF-7 cell growth after 72 h with similar efficiency. 1,25$(OH)_2D_3$, and analogues have little effect on MB-231 (Figure 1B); however, there is a small but significant decrease in cell viability with calcipotriol at 100 and 1000 nM concentrations. Still, the cell viability at 1000 nM was only reduced to 90%, whereas the three compounds reduce MCF-7 cell viability to 60−67%. For MB-468 (Figure 1C), 10 nM of calcipitriol or EB1089 appears to increase cell viability. This is also seen with 1,25$(OH)_2D_3$, but the effect was not statistically significant. All compounds returned MB-468 viability to 95−100% at 1000 nM.

The finding that MCF-7 was sensitive to these compounds but MB-231 and MB-468 were not growth inhibited was not surprising as it mirrors our previous findings.[12] However, we expected EB1089 to be more potent than 1,25$(OH)_2D_3$. EB1089 was previously reported to be more potent than 1,25$(OH)_2D_3$ using MCF-7 cells although the culturing and growth assessment methodology used was much different than ours.[24] The same authors assessed calcipotriol to have similar potency to 1,25$(OH)_2D_3$ in MCF-7 cells, which matches our results here.[23]

We next assessed the effect of PRIMA-1^{MET} on the cell lines alone. PRIMA-1^{MET} has been previously shown to reactivate the R273H mutant of p53 found in MB-468 and was specifically shown to cause an increase in p53 responsive genes such as p21 and PUMA in MB-468.[16,21] It has also been shown to increase levels of *SLC7A11*, a gene suppressed by mutant p53, in MB-231.[21] Because it had already been shown that mutant p53 in our cell lines was reactivated by PRIMA-

https://doi.org/10.1021/acsomega.3c03719
ACS Omega 2023, 8, 30500−30507

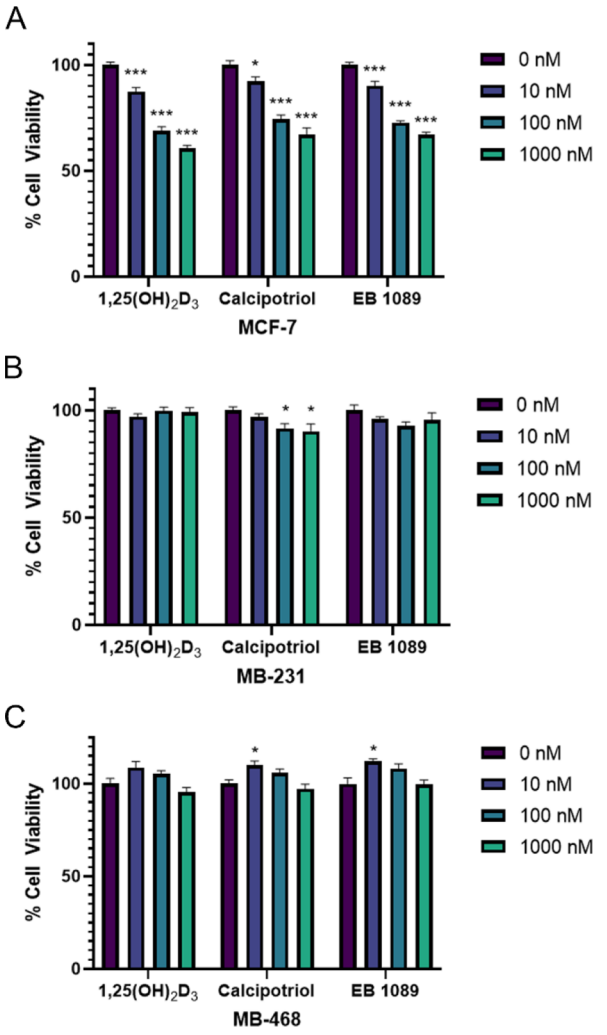


Figure 1. Effect of vitamin D, calcipotriol, and EB1089 on cell viability. MCF-7 (A), MB-231 (B), and MB-468 (C) cultured in stripped-serum media were treated with $1,25(OH)_2D_3$, calcipotriol, or EB1089 for 72 h. Cell viability was measured using CellTiter-Glo. Error bars denote ± SEM. Significant deviation from the 0 nM controls was determined using two-way ANOVA with Dunnett post hoc analysis. * $p < 0.03$, *** $p < 0.001$.

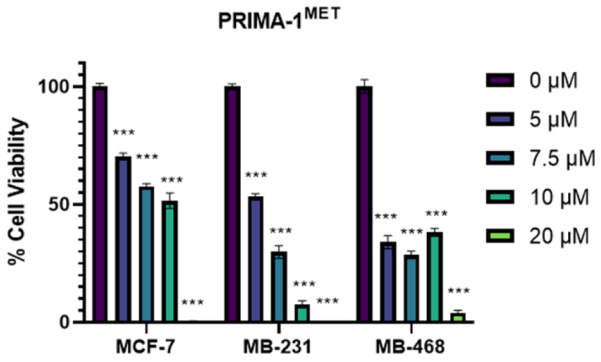


Figure 2. Effect of PRIMA-1^{MET} on breast cancer cell viability. MCF-7, MB-231, and MB-468 cells cultured in stripped-serum media were treated with increasing concentrations of PRIMA-1^{MET} for 72 h. Cell viability was measured using CellTiter-Glo. Error bars denote ± SEM. Significant deviation from the 0 nM controls was determined using two-way ANOVA with Dunnett post hoc analysis. *** $p < 0.001$.

1^{MET}, we decided to use the compound to assess its effect on vitamin D sensitivity in our cell lines. We hypothesized that PRIMA-1^{MET} would cause MB-231 and MB-468 cells to become more sensitive to $1,25(OH)_2D_3$ and its analogues.

PRIMA-1^{MET} killed the three cell lines, but with varied potency (Figure 2). Both MB-231 and MB-468 are more sensitive than MCF-7, with the cell viability falling below 50% at less than 5 μM (MB-468) or between 5 and 7.5 μM (MB-231). MCF-7 cells were just above 50% viability (51%) at 10 μM. PRIMA-1^{MET} shows roughly equal cell viability with MB-468 at 5, 7.5, and 10 μM, while the other cell lines show clear dose−response behavior in this concentration range. All three cell lines have little to no viability at 20 μM PRIMA-1^{MET}. These cell lines seem more sensitive to PRIMA-1^{MET} than when measured by others, which may be attributed to the necessity of using charcoal stripped serum in order to study vitamin D. Normal FBS would be expected to have significant amounts of vitamin D already present, complicating analysis of the effect of the vitamin D or analogues we add to the culture. We also refrained from using sodium pyruvate in our cell culture media for our experiments. Sodium pyruvate has been shown by us and others to neutralize reactive oxygen species in cell culture,[26−28] which can hide the full effect of small molecules like PRIMA-1^{MET} in cell culture.

Amirtharaj et al. report 95% viability of MB-231 cells after 24 h with 50 μM PRIMA-1^{MET}, which is 5X higher than the concentration killing almost all of our MB-231 at 72 h.[17] Synnott et al. reported an IC_{50} value of 31.1 μM for MCF-7 after 5 days incubation, which is far higher than our data (IC_{50} near 10 μM).[21] Their value of ∼2.5 μM for MB-468 is much closer to ours. Makhale et al. determined an IC_{50} of 31.86 μM for MB-231 after 6 days incubation.[29] While these three studies examine cell viability using different methods and time points than ours, they all used media with 10% FBS. It seems likely that this difference is the reason for higher sensitivity to PRIMA-1^{MET} in our study.

We examined the expression level of p53 by immunoblot with increasing concentrations of PRIMA-1^{MET} to determine if the compound caused any difference. The expression levels varied widely between cell lines making it difficult to quantify them on the same gel. As seen in Figure 3A, even when loading substantially more MCF-7 lysate, we detected very small amounts of p53. Overall, while it appears there may be a slight increase in p53 expression in some cell lines at 10 μM PRIMA-1^{MET}, the effect was not statistically significant. We can conclude that the effects of PRIMA-1^{MET} are not caused by an increase in expression level for p53.

We next wanted to determine if PRIMA-1^{MET} would cause MB-231 and MB-468 to become growth inhibited by vitamin D or its analogues, including MCF-7 as our control. For MCF-7, it appears that PRIMA-1^{MET} reduces the ability of $1,25(OH)_2D_3$ to inhibit cell growth (Figure 4A). Without PRIMA-1^{MET}, 1000 nM $1,25(OH)_2D_3$ drops cell viability to 61%, but in the presence of 10 μM PRIMA-1^{MET}, 1000 nM $1,25(OH)_2D_3$ drops cell viability to 81% of the matched control and lesser amounts of $1,25(OH)_2D_3$ have no statistically significant effect (Figure 4A). The results with calcipotriol (Figure 4B) and EB1089 (Figure 4C) look similar, with $1,25(OH)_2D_3$ analogue effectiveness decreasing as PRIMA-1^{MET} concentration increases.

For MB-231, PRIMA-1^{MET} was able to alter cellular response to $1,25(OH)_2D_3$, but not in the direction we hypothesized (Figure 5A). While MB-231 was non-responsive to $1,25(OH)_2D_3$ at 0 and 5 μM PRIMA-1^{MET}, at 7.5 μM there

https://doi.org/10.1021/acsomega.3c03719
ACS Omega 2023, 8, 30500−30507

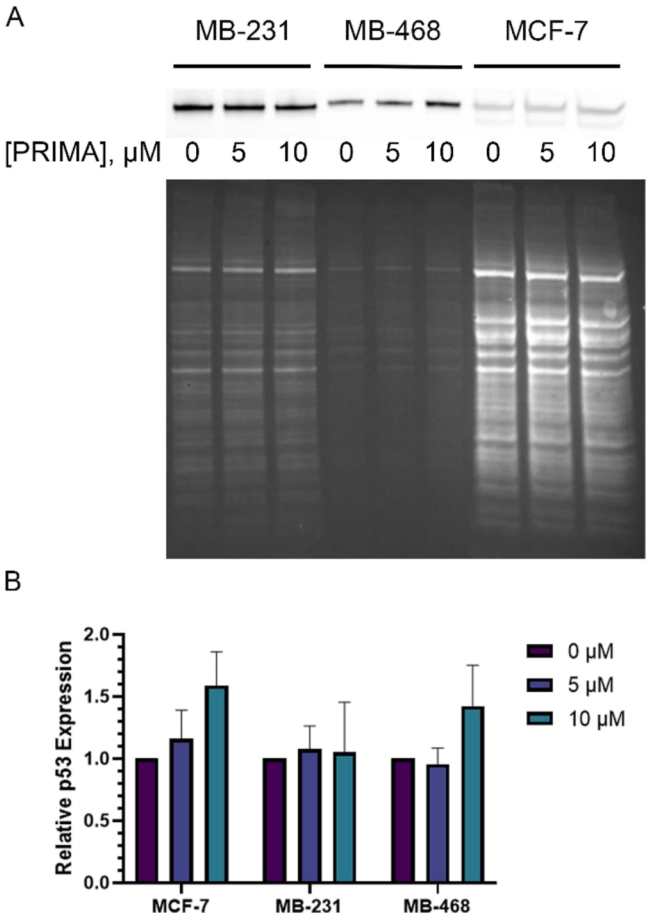


Figure 3. Effect of PRIMA-1^{MET} on p53 expression in breast cancer cells. Cells cultured in stripped-serum media were treated for 24 h with 0, 5, or 10 μM PRIMA-1^{MET}. The cells were lysed in RIPA buffer and analyzed by immunoblot against p53. (A) Representative immunoblot, top, and total protein stain loading control, bottom. (B) Quantitation of relative amounts of p53 for three blots, normalized to the vehicle control for each cell line. Error bars denote ± SEM. None of the treatments were significantly different than vehicle treated cells.

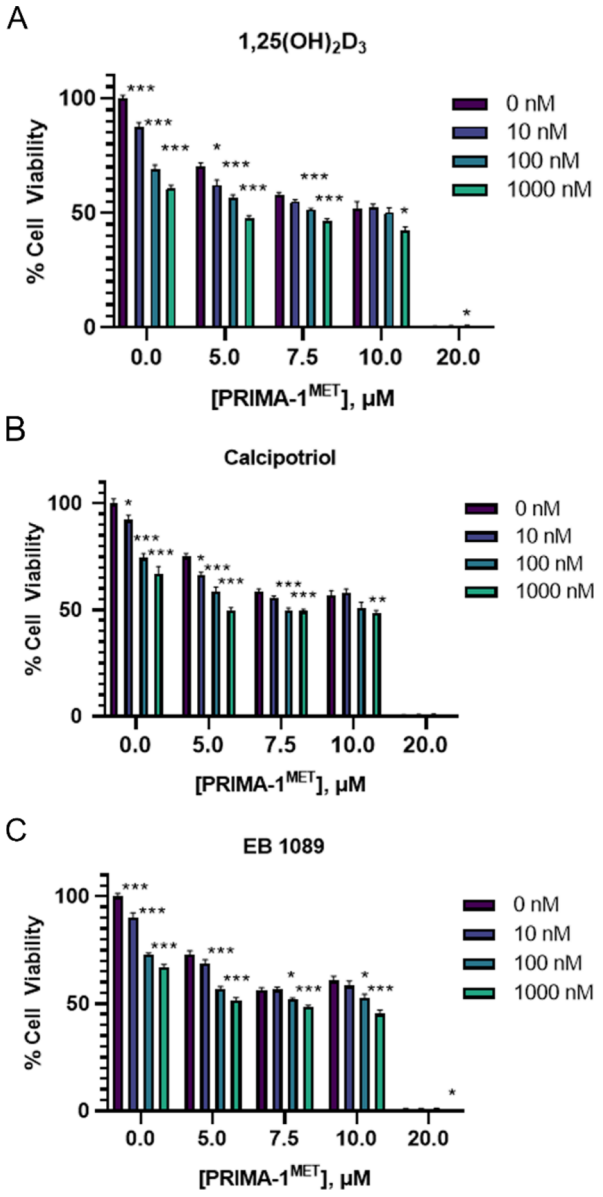


Figure 4. PRIMA-1^{MET} effect on $1,25(OH)_2D_3$, calcipotriol, and EB1089 MCF-7 growth inhibition. MCF-7 cells cultured in stripped-serum media were treated with increasing concentrations of PRIMA-1^{MET} and $1,25(OH)_2D_3$ (A), calcipotriol (B), or EB1089(C) for 72 h. Cell viability was measured using CellTiter-Glo. Error bars denote ± SEM. Significant deviation from the 0 nM controls was determined using two-way ANOVA with Dunnett post hoc analysis. * $p < 0.03$, ** $p < 0.002$, *** $p < 0.001$.

is a small but significant increase in cell viability with 10 or 100 nM $1,25(OH)_2D_3$. This is reminiscent of the increase in cell viability we often see with MB-468. Interestingly, we do not see this happen with either calcipotriol (Figure 5B) or EB1089 (Figure 5C). In the case of calcipotriol, the very slight response without PRIMA-1^{MET} is abolished as PRIMA-1^{MET} concentration increases. For EB1089, there's almost no statistically significant response to any combination of EB1089 and PRIMA-1^{MET}.

For MB-468, both calcipotriol and EB1089 cause a statistically significant increase in cell viability at 10 nM before returning to baseline viability at 1000 nM (Figure 6B,C). A similar pattern is seen with $1,25(OH)_2D_3$, but it does not reach statistical significance (Figure 6A). Increasing amounts of PRIMA-1^{MET} change the response pattern with increasing $1,25(OH)_2D_3$ or analogue; however, PRIMA-1^{MET} does not provide a consistent decrease in cell viability as $1,25(OH)_2D_3$ (or analogue) concentration increases.

Taken together, we conclude that PRIMA-1^{MET} is not able to restore MB-231 or MB-468 cells' sensitivity to vitamin D or vitamin D analogues. This supports the conclusion that mutant p53 is not responsible for the atypical response of these cell lines to vitamin D, proving our initial hypothesis incorrect. At this point, we still do not know why our cells are insensitive to vitamin D nor do we know how to make them sensitive. While we established changes in mRNA levels of CYP24A1, CYB27B1, and the VDR in response to $25\text{-}OHD_3$ and $1,25(OH)_2D_3$ in a previous study,[12] we have yet to determine protein levels by immunoblot or activity of these enzymes/receptors. Cancer cells may upregulate CYP24A1, which catabolizes both $1,25(OH)_2D_3$ and $25\text{-}OHD_3$.[30] This would lead to less available vitamin D to trigger signaling pathways to slow cell growth or promote differentiation. There can also be a downregulation of the CYP27B1 enzyme, which hydroxylates $25\text{-}OHD_3$ to form $1,25(OH)_2D_3$. This could be an explanation

https://doi.org/10.1021/acsomega.3c03719
ACS Omega 2023, 8, 30500−30507

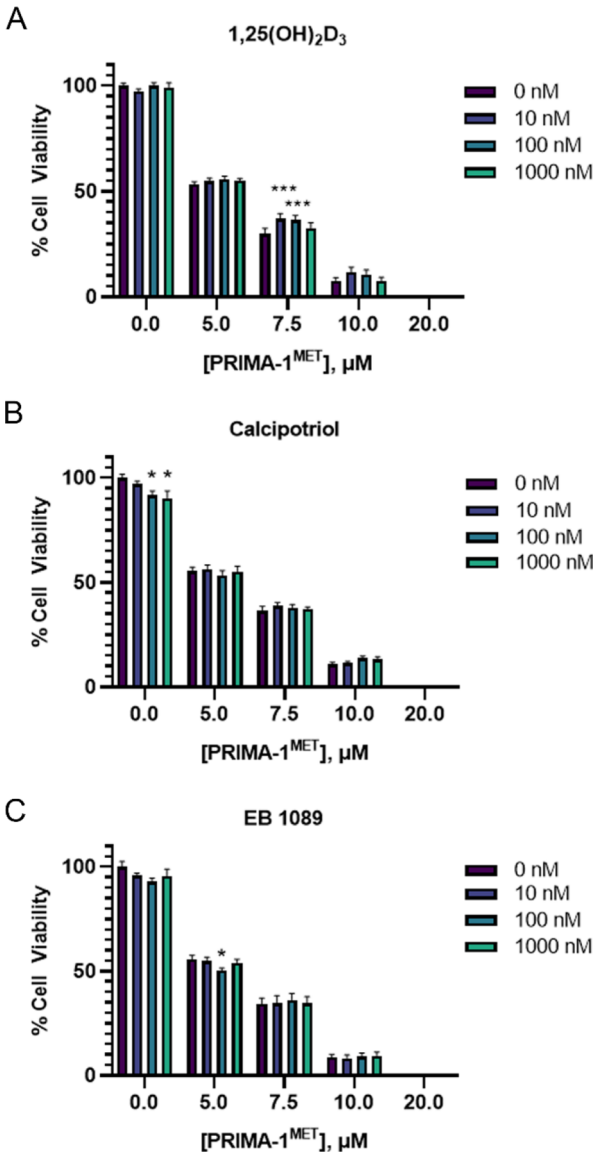


Figure 5. PRIMA-1^{MET} effect on $1,25(OH)_2D_3$, calcipotriol, and EB1089 MB-231 growth inhibition. MB-231 cells cultured in stripped-serum media were treated with increasing concentrations of PRIMA-1^{MET} and $1,25(OH)_2D_3$ (A), calcipotriol (B), or EB1089 (C) for 72 h. Cell viability was measured using CellTiter-Glo. Error bars denote ± SEM. Significant deviation from the 0 nM controls was determined using two-way ANOVA with Dunnett post hoc analysis. * $p < 0.03$, *** $p < 0.001$.

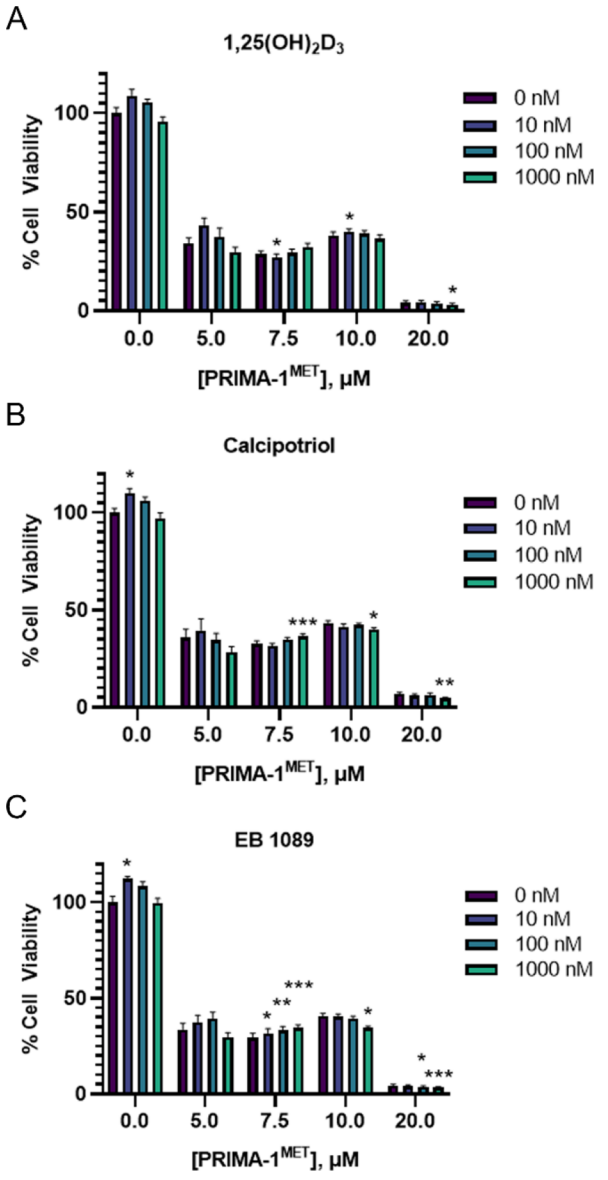


Figure 6. PRIMA-1^{MET} effect on $1,25(OH)_2D_3$, calcipotriol, and EB1089 MB-468 growth inhibition. MB-468 cells cultured in stripped-serum media were treated with increasing concentrations of PRIMA-1^{MET} and $1,25(OH)_2D_3$ (A), calcipotriol (B), or EB1089 (C) for 72 h. Cell viability was measured using CellTiter-Glo. Error bars denote ± SEM. Significant deviation from the 0 nM controls was determined using two-way ANOVA with Dunnett post hoc analysis. * $p < 0.03$, ** $p < 0.002$, *** $p < 0.001$.

for vitamin D insensitivity in vivo, but not in our particular study because we used $1,25(OH)_2D_3$ and analogues, which do not require hydroxylation by CYP27B1.

If CYP24A1 upregulation is an issue in TNBC, targeted inhibition of CYP24A1 may be an option in cancer treatment. Indeed, there are already studies showing inhibition of CYP24A1 with RNAi increasing the $1,25(OH)_2D_3$ antiproliferative effect on colorectal cancer cells.[31] The drug astemizole has been found to be synergistic with vitamin D in hepatocellular carcinoma cells.[32] In addition, astemizole was tested in some breast cancer cells and was found to inhibit CYP24A1 and increase VDR.[33] That study showed there was a synergetic anti-proliferative effect between astemizole and vitamin D. It would be interesting to try this combination in our cell lines, which were not tested in this study, to see if we find increased sensitivity to vitamin D. We could also test if these two drugs could increase the potency of typical chemotherapeutic drugs like paclitaxel in our TNBC cell lines.

There has already been some examination of VDR in MB-231 cells, which were found to express low levels of the receptor. However, Peng et al. found that overexpressing the VDR in MB-231 did not restore sensitivity to a vitamin D analogue.[34] This indicates that low levels of VDR are not responsible for the lack of response to $1,25(OH)_2D_3$. In addition, even though levels of VDR may vary between our cell lines, we have previously shown that treatment of MCF-7, MB-231, and MB-468 with $1,25(OH)_2D_3$ results in significant increases in CYP24A1 RNA,[12] a response that requires functional VDR.[35]

https://doi.org/10.1021/acsomega.3c03719
ACS Omega 2023, 8, 30500−30507

A study published last year implicates the role of estrogen-related receptor α (ERRα) as crucial to vitamin D sensitivity in the TNBC cell line SUM149PT.[36] This study also showed small increases in the estrogen receptor gene and an estrogen synthesis pathway gene in MB-231, but did not further characterize MB-231 response to vitamin D. It was previously known that 100 nM vitamin D would cause re-expression of the estrogen receptor in SUM-229 PE, another TNBC cell line, and in patient derived TNBC cells.[37] Probing expression and function of ERRα as it relates to vitamin D would be another potential future direction.

In regards to PRIMA-1^{MET}, it is slightly more potent in the TNBC cells than in MCF-7, but it does still kill MCF-7 cells even though they express wild-type p53. Recent literature reports multiple mechanisms of action for PRIMA-1^{MET}, both p53 dependent and independent. Numerous reports indicate that PRIMA-1^{MET} causes oxidative stress in cells with both mutant and wild-type p53, including in breast cancer cells.[21,38−44] It was also shown to reactivate wild-type p53 that was inactivated by malignant melanoma cells.[45] Currently, many of the clinical studies with PRIMA-1^{MET} do not have publicly posted data. In one study, researchers tested patients for p53 reactivation during the lead-in portion, with patients showing activation of p53 downstream targets.[46] It seems there is some evidence that PRIMA-1^{MET} is activating mutant p53 in vivo.

Another possible mechanism of cell death for PRIMA-1^{MET} is ferroptosis, an iron dependent form of cell death related to oxidative status of the cell.[47−49] Another future research direction could be probing the non-p53-dependent mechanisms after PRIMA-1^{MET} treatment in TNBC cells and MCF-7. As has been shown in other cell types, we could try to block PRIMA-1^{MET} activity with *N*-acetylcysteine (NAC) or enhance it with buthionine sulfoximine (BSO) to probe the effect of ROS in the killing of our cells. We could also try blocking the effects of PRIMA-1^{MET} with commercially available ferroptosis inhibitors, such as ferrostatin-1 or deferoxamine.

AUTHOR INFORMATION

Corresponding Author

Jessica L. Kelts − *Department of Natural Sciences, University of Michigan-Flint, Flint, Michigan 48502, United States;* orcid.org/0000-0002-1084-0498; Email: jkelts@umich.edu

Author

Shadi N. Kotob − *Department of Natural Sciences, University of Michigan-Flint, Flint, Michigan 48502, United States*

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.3c03719

Author Contributions

S.N.K. contributed to this work through development of method for all experiments, generation of data for publication, and writing and editing of the draft. J.L.K. contributed to this work through initial conception, funding acquisition, acquiring data, creating figures, initial draft writing, and final editing of the manuscript. All authors have given approval to the final version of the manuscript.

Funding

This research was funded by the Ben F. Bryer Chemistry and Biochemistry Faculty Fellow Award and the UM-Flint Reconnect2Scholarship Award, both to J.L.K. Student research time was supported by the UM-Flint Undergraduate Research Opportunity Fund (S.N.K.).

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We would like to thank the Dr. Ben F. Bryer Medical Research Foundation for their continuing financial support through both the Bryer Instrumentation fund and the Chemistry and Biochemistry Faculty Fellow Award to J.L.K. We would like to thank UM-Flint, the Chancellor's office, and the Office of Research for their research and financial support, especially the Reconnect2Scholarship award to J.L.K. and Undergraduate Research Opportunity Program (UROP) funding to S.N.K. We would like to thank Ashlyn Savoie for assistance with cell culture. We would like to thank Dennis Viele for help with statistical analysis of our data. We would like to thank Kerigan Condron, Breann Hewitt, and Dominika Lazarska for laboratory and ordering support.

ABBREVIATIONS

$1,25(OH)_2D_3$, 1,25-dihydroxycholecalciferol; $25\text{-}OHD_3$, 25-hydroxycholecalciferol; NAC, *N*-acetylcysteine; BCA, biocinchoninic acid; BSO, DL-buthionine-(*S*,*R*)-sulfoximine; CYP24A1, cytochrome P450 family 24 subfamily A member 1; CYP27B1, cytochrome P450 family 27 subfamily B member 1; DMSO, dimethylsulfoxide; ERRα, estrogen-related receptor α; EDTA, ethylenediaminetetraacetic acid; FBS, fetal bovine serum; HER2, human epidermal growth factor receptor 2; HRP, horseradish peroxidase; MES, 2-(*N*-morpholino)-ethanesulfonic acid; PBS, phosphate buffered saline; PRIMA-1^{MET}, p53 reactivation and induction of massive apoptosis (methylated form); PVDF, polyvinylidene fluoride; RIPA, radioimmunoprecipitation assay; SDS, sodium dodecyl sulfate; TNBC, triple-negative breast cancer; VDR, vitamin D receptor

REFERENCES

(1) Giaquinto, A. N.; Sung, H.; Miller, K. D.; Kramer, J. L.; Newman, L. A.; Minihan, A.; Jemal, A.; Siegel, R. L. Breast Cancer Statistics, 2022. *CA Cancer J. Clin.* **2022**, *72*, 524−541.

(2) Bou Zerdan, M.; Ghorayeb, T.; Saliba, F.; Allam, S.; Bou Zerdan, M.; Yaghi, M.; Bilani, N.; Jaafar, R.; Nahleh, Z. Triple Negative Breast Cancer: Updates on Classification and Treatment in 2021. *Cancers (Basel)* **2022**, *14*, 1253.

(3) Osborne, C. K. Tamoxifen in the treatment of breast cancer. *N. Engl. J. Med.* **1998**, *339*, 1609−1618.

(4) Vu, T.; Claret, F. X. Trastuzumab: updated mechanisms of action and resistance in breast cancer. *Front. Oncol.* **2012**, *2*, 62.

(5) Welsh, J. Vitamin D and Breast Cancer: Mechanistic Update. *JBMR Plus* **2021**, *5*, No. e10582.

(6) Grant, W. B. Review of Recent Advances in Understanding the Role of Vitamin D in Reducing Cancer Risk: Breast, Colorectal, Prostate, and Overall Cancer. *Anticancer Res.* **2020**, *40*, 491−499.

(7) Lope, V.; Castelló, A.; Mena-Bravo, A.; Amiano, P.; Aragonés, N.; Fernández-Villa, T.; Guevara, M.; Dierssen-Sotos, T.; Fernandez-Tardón, G.; Castaño-Vinyals, G.; Marcos-Gragera, R.; Moreno, V.; Salas-Trejo, D.; Diaz-Santos, M.; Oribe, M.; Romieu, I.; Kogevinas, M.; Priego-Capote, F.; Pérez-Gómez, B.; Pollán, M. Serum 25-hydroxyvitamin D and breast cancer risk by pathological subtype (MCC-Spain). *J. Steroid Biochem. Mol. Biol.* **2018**, *182*, 4−13.

(8) Qin, B.; Xu, B.; Ji, N.; Yao, S.; Pawlish, K.; Llanos, A. A. M.; Lin, Y.; Demissie, K.; Ambrosone, C. B.; Hong, C. C.; et al. Intake of vitamin D and calcium, sun exposure, and risk of breast cancer subtypes among black women. *Am. J. Clin. Nutr.* **2020**, *111*, 396−405.

(9) Soljic, M.; Mrklic, I.; Tomic, S.; Omrcen, T.; Sutalo, N.; Bevanda, M.; Vrdoljak, E. Prognostic value of vitamin D receptor and insulin-like growth factor receptor 1 expression in triple-negative breast cancer. *J. Clin. Pathol.* **2018**, *71*, 34−39.
(10) Huss, L.; Butt, S. T.; Borgquist, S.; Elebro, K.; Sandsveden, M.; Rosendahl, A.; Manjer, J. Vitamin D receptor expression in invasive breast tumors and breast cancer survival. *Breast Cancer Res.* **2019**, *21*, 84.
(11) LaPorta, E.; Welsh, J. Modeling vitamin D actions in triple negative/basal-like breast cancer. *J. Steroid Biochem. Mol. Biol.* **2014**, *144*, 65−73.
(12) Richards, S. E.; Weierstahl, K. A.; Kelts, J. L. Vitamin D effect on growth and vitamin D metabolizing enzymes in triple-negative breast cancer. *Anticancer Res.* **2015**, *35*, 805−810.
(13) Stambolsky, P.; Tabach, Y.; Fontemaggi, G.; Weisz, L.; Maor-Aloni, R.; Shiff, I.; Kogan, I.; Shay, M.; Kalo, E.; et al. Modulation of the vitamin D3 response by cancer-associated mutant p53. *Cancer Cell* **2010**, *17*, 273−285.
(14) Wilhelm, C. A.; Clor, Z. J.; Kelts, J. L. Effect of Vitamin D on Paclitaxel Efficacy in Triple-negative Breast Cancer Cell Lines. *Anticancer Res.* **2018**, *38*, 5043−5048.
(15) Lambert, J. M. R.; Gorzov, P.; Veprintsev, D. B.; Söderqvist, M.; Segerbäck, D.; Bergman, J.; Fersht, A. R.; Hainaut, P.; Wiman, K. G.; Bykov, V. J. N. PRIMA-1 reactivates mutant p53 by covalent binding to the core domain. *Cancer Cell* **2009**, *15*, 376−388.
(16) Bykov, V. J. N.; Issaeva, N.; Shilov, A.; Hultcrantz, M.; Pugacheva, E.; Chumakov, P.; Bergman, J.; Wiman, K. G.; Selivanova, G. Restoration of the tumor suppressor function to mutant p53 by a low-molecular-weight compound. *Nat. Med.* **2002**, *8*, 282−288.
(17) Amirtharaj, F.; Venkatesh, G. H.; Wojtas, B.; Nawafleh, H. H.; Mahmood, A. S.; Nizami, Z. N.; Khan, M. S.; Thiery, J.; Chouaib, S. p53 reactivating small molecule PRIMA-1(MET)/APR-246 regulates genomic instability in MDA-MB-231 cells. *Oncol. Rep.* **2022**, *47*, 85.
(18) Izetti, P.; Hautefeuille, A.; Abujamra, A. L.; de Farias, C. B.; Giacomazzi, J.; Alemar, B.; Lenz, G.; Roesler, R.; Schwartsmann, G.; Osvaldt, A. B.; et al. PRIMA-1, a mutant p53 reactivator, induces apoptosis and enhances chemotherapeutic cytotoxicity in pancreatic cancer cell lines. *Invest. New Drugs* **2014**, *32*, 783−794.
(19) Hollestelle, A.; Nagel, J. H.; Smid, M.; Lam, S.; Elstrodt, F.; Wasielewski, M.; Ng, S. S.; French, P. J.; Peeters, J. K.; Rozendaal, M. J.; Riaz, M.; Koopman, D. G.; ten Hagen, T. L. M.; de Leeuw, B. H. C. G. M.; Zwarthoff, E. C.; Teunisse, A.; van der Spek, P. J.; Klijn, J. G. M.; Dinjens, W. N. M.; Ethier, S. P.; Clevers, H.; Jochemsen, A. G.; den Bakker, M. A.; Foekens, J. A.; Martens, J. W. M.; Schutte, M. Distinct gene mutation profiles among luminal-type and basal-type breast cancer cell lines. *Breast Cancer Res. Treat.* **2010**, *121*, 53−64.
(20) Wasielewski, M.; Elstrodt, F.; Klijn, J. G. M.; Berns, E. M. J. J.; Schutte, M. Thirteen new p53 gene mutants identified among 41 human breast cancer cell lines. *Breast Cancer Res. Treat.* **2006**, *99*, 97−101.
(21) Synnott, N. C.; Madden, S. F.; Bykov, V. J. N.; Crown, J.; Wiman, K. G.; Duffy, M. J. The Mutant p53-Targeting Compound APR-246 Induces ROS-Modulating Genes in Breast Cancer Cells. *Transl. Oncol.* **2018**, *11*, 1343−1349.
(22) Knutson, J. C.; LeVan, L. W.; Valliere, C. R.; Bishop, C. W. Pharmacokinetics and systemic effect on calcium homeostasis of 1 alpha,24-dihydroxyvitamin D2 in rats. Comparison with 1 alpha,25-dihydroxyvitamin D2, calcitriol, and calcipotriol. *Biochem. Pharmacol.* **1997**, *53*, 829−837.
(23) Colston, K. W.; Chander, S. K.; Mackay, A. G.; Coombes, R. C. Effects of synthetic vitamin D analogues on breast cancer cell proliferation in vivo and in vitro. *Biochem. Pharmacol.* **1992**, *44*, 693−702.
(24) Colston, K. W.; Mackay, A. G.; James, S. Y.; Binderup, L.; Chander, S.; Coombes, R. C. EB1089: a new vitamin D analogue that inhibits the growth of breast cancer cells in vivo and in vitro. *Biochem. Pharmacol.* **1992**, *44*, 2273−2280.
(25) Valrance, M. E.; Brunet, A. H.; Welsh, J. Vitamin D receptor-dependent inhibition of mammary tumor growth by EB1089 and ultraviolet radiation in vivo. *Endocrinology* **2007**, *148*, 4887−4894.
(26) Kelts, J. L.; Cali, J. J.; Duellman, S. J.; Shultz, J. Altered cytotoxicity of ROS-inducing compounds by sodium pyruvate in cell culture medium depends on the location of ROS generation. *Springerplus* **2015**, *4*, 269.
(27) Giandomenico, A. R.; Cerniglia, G. E.; Biaglow, J. E.; Stevens, C. W.; Koch, C. J. The importance of sodium pyruvate in assessing damage produced by hydrogen peroxide. *Free Radic. Biol. Med.* **1997**, *23*, 426−434.
(28) Long, L. H.; Halliwell, B. Artefacts in cell culture: pyruvate as a scavenger of hydrogen peroxide generated by ascorbate or epigallocatechin gallate in cell culture media. *Biochem. Biophys. Res. Commun.* **2009**, *388*, 700−704.
(29) Makhale, A.; Nanayakkara, D.; Raninga, P.; Khanna, K. K.; Kalimutho, M. CX-5461 Enhances the Efficacy of APR-246 via Induction of DNA Damage and Replication Stress in Triple-Negative Breast Cancer. *Int. J. Mol. Sci.* **2021**, *22*, 5782.
(30) Welsh, J. Cellular and molecular effects of vitamin D on carcinogenesis. *Arch. Biochem. Biophys.* **2012**, *523*, 107−114.
(31) Sun, H.; Jiang, C.; Cong, L.; Wu, N.; Wang, X.; Hao, M.; Liu, T.; Wang, L.; Liu, Y.; Cong, X. CYP24A1 Inhibition Facilitates the Antiproliferative Effect of 1,25(OH)(2)D(3) Through Downregulation of the WNT/beta-Catenin Pathway and Methylation-Mediated Regulation of CYP24A1 in Colorectal Cancer Cells. *DNA Cell Biol.* **2018**, *37*, 742−749.
(32) Xu, J.; Wang, Y.; Zhang, Y.; Dang, S.; He, S. Astemizole promotes the anti-tumor effect of vitamin D through inhibiting miR-125a-5p-meidated regulation of VDR in HCC. *Biomed. Pharmacother.* **2018**, *107*, 1682−1691.
(33) García-Quiroz, J.; García-Becerra, R.; Barrera, D.; Santos, N.; Avila, E.; Ordaz-Rosado, D.; Rivas-Suárez, M.; Halhali, A.; Rodríguez, P.; Gamboa-Domínguez, A.; Medina-Franco, H.; Camacho, J.; Larrea, F.; Díaz, L. Astemizole synergizes calcitriol antiproliferative activity by inhibiting CYP24A1 and upregulating VDR: a novel approach for breast cancer therapy. *PLoS One* **2012**, *7*, No. e45063.
(34) Peng, X.; Jhaveri, P.; Hussain-Hakimjee, E. A.; Mehta, R. G. Overexpression of ER and VDR is not sufficient to make ER-negative MDA-MB231 breast cancer cells responsive to 1alpha-hydroxyvitamin D5. *Carcinogenesis* **2007**, *28*, 1000−1007.
(35) Takeyama, K.; Kitanaka, S.; Sato, T.; Kobori, M.; Yanagisawa, J.; Kato, S. 25-Hydroxyvitamin D3 1alpha-hydroxylase and vitamin D synthesis. *Science* **1997**, *277*, 1827−1830.
(36) Danza, K.; Porcelli, L.; De Summa, S.; Di Fonte, R.; Pilato, B.; Lacalamita, R.; Serrati, S.; Azzariti, A.; Tommasi, S. The ERRalpha-VDR axis promotes calcitriol degradation and estrogen signaling in breast cancer cells, while VDR-CYP24A1-ERRalpha overexpression correlates with poor prognosis in patients with basal-like breast cancer. *Mol. Oncol.* **2022**, *16*, 904−920.
(37) Santos-Martínez, N.; Díaz, L.; Ordaz-Rosado, D.; García-Quiroz, J.; Barrera, D.; Avila, E.; Halhali, A.; Medina-Franco, H.; Ibarra-Sánchez, M. J.; Esparza-López, J.; Camacho, J.; Larrea, F.; García-Becerra, R. Calcitriol restores antiestrogen responsiveness in estrogen receptor negative breast cancer cells: a potential new therapeutic approach. *BMC Cancer* **2014**, *14*, 230.
(38) Ali, D.; Mohammad, D. K.; Mujahed, H.; Jonson-Videsäter, K.; Nore, B.; Paul, C.; Lehmann, S. Anti-leukaemic effects induced by APR-246 are dependent on induction of oxidative stress and the NFE2L2/HMOX1 axis that can be targeted by PI3K and mTOR inhibitors in acute myeloid leukaemia cells. *Br. J. Haematol.* **2016**, *174*, 117−126.
(39) Perdrix, A.; Najem, A.; Saussez, S.; Awada, A.; Journe, F.; Ghanem, G.; Krayem, M. PRIMA-1 and PRIMA-1(Met) (APR-246): From Mutant/Wild Type p53 Reactivation to Unexpected Mechanisms Underlying Their Potent Anti-Tumor Effect in Combinatorial Therapies. *Cancers (Basel)* **2017**, *9*, 172.
(40) Kobayashi, T.; Makino, T.; Yamashita, K.; Saito, T.; Tanaka, K.; Takahashi, T.; Kurokawa, Y.; Yamasaki, M.; Nakajima, K.; Morii, E.;

https://doi.org/10.1021/acsomega.3c03719
ACS Omega 2023, 8, 30500−30507

et al. APR-246 induces apoptosis and enhances chemo-sensitivity via activation of ROS and TAp73-Noxa signal in oesophageal squamous cell cancer with TP53 missense mutation. *Br. J. Cancer* **2021**, *125*, 1523−1532.
(41) Yoshikawa, N.; Kajiyama, H.; Nakamura, K.; Utsumi, F.; Niimi, K.; Mitsui, H.; Sekiya, R.; Suzuki, S.; Shibata, K.; Callen, D.; et al. PRIMA-1MET induces apoptosis through accumulation of intracellular reactive oxygen species irrespective of p53 status and chemosensitivity in epithelial ovarian cancer cells. *Oncol. Rep.* **2016**, *35*, 2543−2552.
(42) Grellety, T.; Laroche-Clary, A.; Chaire, V.; Lagarde, P.; Chibon, F.; Neuville, A.; Italiano, A. PRIMA-1(MET) induces death in soft-tissue sarcomas cell independent of p53. *BMC Cancer* **2015**, *15*, 684.
(43) Tessoulin, B.; Descamps, G.; Moreau, P.; Maïga, S.; Lodé, L.; Godon, C.; Marionneau-Lambot, S.; Oullier, T.; Le Gouill, S.; Amiot, M.; Pellat-Deceunynck, C. PRIMA-1Met induces myeloma cell death independent of p53 by impairing the GSH/ROS balance. *Blood* **2014**, *124*, 1626−1636.
(44) Fujihara, K. M.; Corrales Benitez, M.; Cabalag, C. S.; Zhang, B. Z.; Ko, H. S.; Liu, D. S.; Simpson, K. J.; Haupt, Y.; Lipton, L.; Haupt, S.; et al. SLC7A11 Is a Superior Determinant of APR-246 (Eprenetapopt) Response than TP53 Mutation Status. *Mol. Cancer Ther.* **2021**, *20*, 1858−1867.
(45) Bao, W.; Chen, M.; Zhao, X.; Kumar, R.; Spinnler, C.; Thullberg, M.; Issaeva, N.; Selivanova, G.; Stromblad, S. PRIMA-1Met/APR-246 induces wild-type p53-dependent suppression of malignant melanoma tumor growth in 3D culture and in vivo. *Cell Cycle* **2011**, *10*, 301−307.
(46) Sallman, D. A.; DeZern, A. E.; Garcia-Manero, G.; Steensma, D. P.; Roboz, G. J.; Sekeres, M. A.; Cluzeau, T.; Sweet, K. L.; McLemore, A.; McGraw, K. L.; et al. Eprenetapopt (APR-246) and Azacitidine in TP53-Mutant Myelodysplastic Syndromes. *J. Clin. Oncol.* **2021**, *39*, 1584−1594.
(47) Hong, Y.; Ren, T.; Wang, X.; Liu, X.; Fei, Y.; Meng, S.; Han, X.; Sun, C.; Shen, H.; Li, L.; et al. APR-246 triggers ferritinophagy and ferroptosis of diffuse large B-cell lymphoma cells with distinct TP53 mutations. *Leukemia* **2022**, *36*, 2269−2280.
(48) Birsen, R.; Larrue, C.; Decroocq, J.; Johnson, N.; Guiraud, N.; Gotanegre, M.; Cantero-Aguilar, L.; Grignano, E.; Huynh, T.; Fontenay, M.; et al. APR-246 induces early cell death by ferroptosis in acute myeloid leukemia. *Haematologica* **2022**, *107*, 403−416.
(49) Wang, Z.; Hu, H.; Heitink, L.; Rogers, K.; You, Y.; Tan, T.; Suen, C. L. W.; Garnham, A.; Chen, H.; Lieschke, E.; et al. The anti-cancer agent APR-246 can activate several programmed cell death processes to kill malignant cells. *Cell Death Differ.* **2023**, *30*, 1033−1046.

https://doi.org/10.1021/acsomega.3c03719
ACS Omega 2023, 8, 30500−30507