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Article

Sensitization of Resistant Breast Cancer Cells with a Jumonji Family Histone Demethylase Inhibitor

Balraj Singh[1,2,*], Vanessa Nicole Sarli[1,2] and Anthony Lucci[1,2,*]

[1] Department of Breast Surgical Oncology, The University of Texas MD Anderson Cancer Center, Houston, Texas, USA
[2] Morgan Welch Inflammatory Breast Cancer Research Program and Clinic, The University of Texas MD Anderson Cancer Center, Houston, Texas, USA
* Correspondence: alucci@mdanderson.org (A.L.), bsingh@mdanderson.org (B.S.)

Simple Summary: Using a cell culture model of resistant breast cancer cells with the phenotype that is often responsible for early relapse of triple-negative breast cancer, namely, persistence of these cells in reversible quiescence under a variety of challenges, we found that reprogramming of the epigenome by treatment with JIB-04, a small-molecule inhibitor of Jumonji family histone demethylases, sensitized resistant cells. We used this model of deep intrinsic resistance featuring many molecular mechanisms of achieving this phenotype to perform lengthy evaluations of less cytotoxic doses of JIB-04. We found that resistant cells derived from triple-negative inflammatory breast cancer cell lines were either much more sensitive to JIB-04 than was the parental cell line or altered by the treatment such that they became sensitive to the chemotherapeutic drugs paclitaxel and doxorubicin. Notably, JIB-04 exposure increased PD-L1 expression in cancer cells, which means that JIB-04 may have clinical application in improving responses of triple-negative breast cancer to anti-PD-L1 therapy.

Abstract: We previously described a model of deep intrinsic resistance of breast cancer wherein we used a function-based approach to selection of cancer cells that can survive a variety of challenges in prolonged but reversible quiescence. Our experimental results suggested that resistant cancer cells possess a variety of mechanisms, including modifications of the epigenome and transcriptome, for generating a high degree of cellular heterogeneity. In the present study, we evaluated JIB-04, a small-molecule epigenetic inhibitor initially discovered to inhibit cancer growth, to determine its ability to affect deep intrinsic resistance in our breast cancer model. We found that long pretreatment with JIB-04 sensitized resistant triple-negative inflammatory breast cancer cells and their parental cell line SUM149 to the chemotherapeutic drugs doxorubicin and paclitaxel. Resistant cancer cells derived from another inflammatory breast cancer cell line, FC-IBC02, were considerably more sensitive to JIB-04 than was the parental cell line. Investigating a mechanism of sensitization, we found that JIB-04 exposure increased the expression of PD-L1 in resistant cells, suggesting that JIB-04 may also sensitize resistant breast cancer cells to anti-PD-L1 immune therapy. Finally, these results support the usefulness of our experimental strategy for evaluating anticancer agents such as JIB-04 that may halt cancer evolution and prevent development of cancer resistance to currently used therapies.

Keywords: resistant TNBC; intra-tumoral heterogeneity; breast cancer relapse; breast cancer epigenome; metastasis prevention; intrinsic resistance of cancer; tumor adaptability; targeting resistant cancer

1. Introduction

Our main study interest is developing therapies for difficult-to-treat breast cancers such as inflammatory breast cancer (IBC). To briefly describe the difficulties in facing this task, we must first examine the nature of the disease and possible ways of halting its development in a timely manner before it advances to clinical metastasis. Development of

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cancer is often a lengthy process that exploits powerful mechanisms that shape organismal evolution [1-5]. The body's defense mechanisms for dealing with rogue cells engage with and successfully control the growth of evolving cancer cells until they are finally unable control. The deep intrinsic treatment resistance of cancer is governed by a small subpopulation of progenitor-like cancer cells that acquire multiple abnormalities, including not only gene mutations but also alterations of the epigenome and transcriptome. Significantly, these cells are effective at opportunistically switching to quiescence to survive a variety of challenges in the body, including current therapies that primarily inhibit the growth of or kill actively proliferating cancer cells. Such resistant cells persist in the body as minimal residual disease [6-8].

To model this phenotype in a cell culture model suitable for therapeutic evaluation, we previously described a function-based selection approach wherein lack of a nutrient (e.g., glutamine) results in massive cell killing. About 0.01% of SUM149 triple-negative IBC (TN-IBC) cells survived in quiescence for several weeks without glutamine and then began proliferating indefinitely [9]. This simple approach is good at modeling deep intrinsic resistance. One benefit of this model is that it can be used for a realistic, lengthy evaluation of less cytotoxic therapeutic agents [9-14]. This is significant because the resistant cells selected in this manner also produce progeny cells over time that proliferate well without being very adaptable. This reflects balance in a living system for maintaining both persistence and proliferation of cells. As an example of therapeutic evaluation performed to assess deep intrinsic resistance in this system, while evaluating resistance to chemotherapeutic drugs, we treat cells with drugs long enough (5-7 days) to kill the majority of proliferative cells. We then assess rare cells that survive in quiescence by observing their growth into colonies upon withdrawing treatment with the chemotherapeutic drugs. We refer resistant cell lines as being metabolically adaptable (MA) based on a selection criterion, although they are adaptable according to multiple criteria. The reason for this lies in a close link between the metabolic and regulatory states [15,16].

We found that TN-IBC–derived cell lines yield adaptable cancer cells that are very robust in switching back and forth between quiescence and proliferation, which validates our approach to modeling deep intrinsic resistance. In contrast, non-IBC triple-negative breast cancer (TNBC) cell lines such as MB-MDA-231 and their metastatic variants selected from two rounds of bone metastases in nude mice after cardiac inoculation of cancer cells yielded cells that could persist in quiescence but failed to grow as healthy cell cultures for an indefinite period [9]. This phenotypic difference between IBC and non-IBC cells, considered in the context of cancer progression, explains the basis for early rather than late relapse of IBC [17,18].

In the present study, we evaluated the ability of JIB-04, a pan-inhibitor of Jumonji family histone demethylases, to affect intrinsic resistance in our model of adaptable TN-IBC cells. Wang et al. identified JIB-04 as an epigenetic modulator in a cancer-selective manner using a cell-based screen [19]. JIB-04 inhibits cancer cell growth *in vitro* and *in vivo*. It has also increased survival durations in mice bearing aggressive 4T1 breast cancer xenografts [19]. Subsequent studies showed that JIB-04 inhibited resistant cancer cells for several cancer types [20-26], making it a desirable small-molecule inhibitor. We found that resistant cancer cells were more sensitive to JIB-04 than was the parental cell line and that long treatment with JIB-04 sensitized the resistant cancer cells to chemotherapeutic drugs, demonstrating the clinical potential of this inhibitor.

2. Materials and Methods

2.1. Cell lines and drugs

A resistant TN-IBC cell line used in this study was SUM149-MA, which was derived from a firefly luciferase-transfected SUM149 cell line (SUM149-Luc) [27]. The resistant TN-IBC cell line FC-IBC02-MA, which was derived from the FC-IBC02 cell line [28], was also used. The generation and characterization of these cell lines and their culture conditions were described previously [9,13]. Because the selection of MA cell lines was performed

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using a medium lacking glutamine, dialyzed fetal bovine serum (FBS) was used instead of regular FBS to further reduce glutamine levels in culture medium. Therefore, all experiments were performed using media containing dialyzed FBS for consistency, even when a medium was supplemented with glutamine (parental cell lines and MA cell lines after the initial selection in the absence of glutamine).

JIB-04 was purchased from Selleck Chemicals (Houston, TX, USA), and paclitaxel and doxorubicin were purchased from Sigma-Aldrich (St. Louis, MO, USA). JIB-04, paclitaxel, and doxorubicin were dissolved in dimethyl sulfoxide (DMSO). Equal volumes of DMSO without drugs was added to the cultures of control dishes. Each solvent volume was no more than 0.04% of the volume of the culture medium.

2.2. Western blotting

Western blotting to detect protein bands as enhanced chemoluminescence signals on x-ray films was performed as described previously [29]. Primary anti-PD-L1 (catalog number 13684; Cell Signaling Technology, Danvers, MA, USA) and anti-HSP90 (catalog number 4875; Cell Signaling Technology) antibodies were used for protein detection. After detection of PD-L1, Western blot membranes were re-probed to detect HSP90, which served as an internal control for normalization of protein loading. Each Western blot was performed at least twice. The relative intensities of the protein bands detected on x-ray films were quantified using ImageJ software (version 153; National Institutes of Health, Bethesda, MD, USA).

2.3. JIB-04 treatment of resistant cancer cells

Resistant and parental TN-IBC cells were treated in parallel with JIB-04 at 62.5-250.0 nM for various periods. The effects of this treatment were manifested in two different ways: 1) massive cell killing followed by growth of colonies in the presence of JIB-04 and 2) massive cell killing with the requirement of removal of JIB-04 for the recovery of remaining viable cells and their growth into colonies. The effects of these treatments on cell growth and morphology were monitored frequently under a microscope. At the end of treatment, the culture dishes containing colonies were stained with crystal violet and photographed or scanned. For quantitation of the relative cell masses in the stained dishes, the colonies were counted.

2.4. Assay of relative resistance of cells to paclitaxel and doxorubicin

To determine whether treatment with JIB-04 affected the sensitivity of the resistant TN-IBC cells to the chemotherapeutic drugs, drug-treated cells were allowed to recover for a few days and then passaged. These JIB-04-treated cells were treated in parallel with control vehicle-treated cells for 5-8 days with predetermined concentrations of chemotherapeutic drugs (5 nM paclitaxel and 100 nM doxorubicin) expected to kill 99% of the proliferating cells. The chemotherapeutic drugs were then removed by changing medium, and surviving cells were allowed to form colonies for 10-12 days. Colonies were stained with crystal violet and counted.

3. Results

3.1. JIB-04 resistance of SUM149-MA cells

To evaluate the resistance to JIB-04 of SUM149-MA cells relative to that of parental SUM149-Luc cells, we treated cultures of both cell lines with different concentrations of JIB-04 and observed them under a microscope. We changed the drug medium as needed to remove floating dead cells from the culture dishes. Typically, when most of the cells were killed and about 1% of the cells were still attached to dishes, we shifted the cells to fresh medium without JIB-04 and let the resistant cells grow into colonies to evaluate the relative resistance of these cells to JIB-04 in both cultures. In these experiments, we consistently observed considerably more colonies in SUM149-MA cultures than in SUM149-Luc control cultures. Figure 1 shows the results of a representative experiment in which

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we treated SUM149-MA and SUM149-Luc cells with 250 nM JIB-04 for 18 days and then allowed the remaining resistant cells to recover and grow into colonies in the absence of JIB-04 for 14 days. The figure shows about 50 SUM149-MA cell colonies of various sizes; the culture of control parental SUM149-Luc cells treated with JIB-4 in parallel did not yield any colonies. This result is similar to the results obtained for most agents, including the chemotherapeutic drugs doxorubicin and paclitaxel, demonstrating the highly adaptable and resistant nature of these cells [9-14].

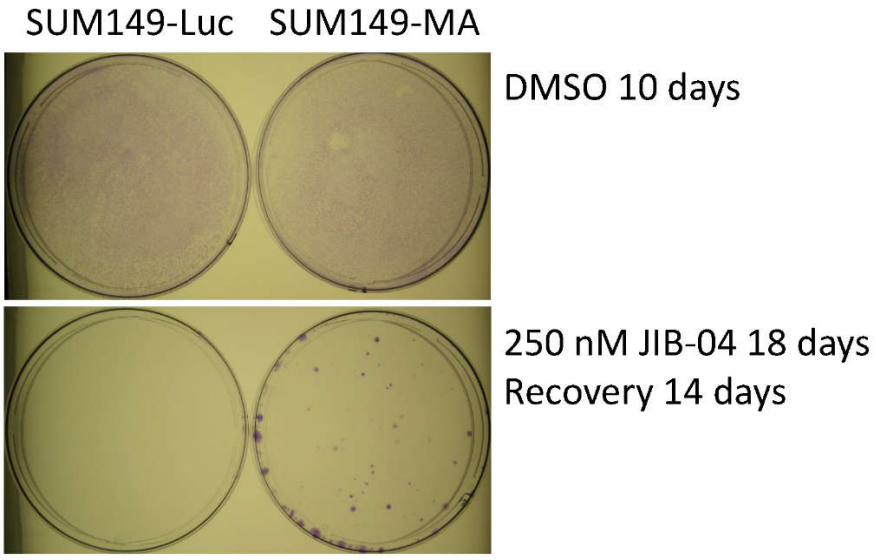

Figure 1. Resistance of SUM149-MA cells to treatment with JIB-04. SUM149-MA and parental SUM149-Luc cells were treated in parallel with 250 nM JIB-04 for 18 days (treatment killed most of the cells) and then allowed to recover and grow into colonies in a drug-free medium for 14 days before staining of the colonies (bottom). The top panel shows input control dishes from treatment of cell cultures with the solvent DMSO for 10 days, which was when both cell cultures grew to comparable confluency as indicated by crystal violet staining.

3.2. Sensitization of SUM149-MA cells to chemotherapeutic drugs by treatment with JIB-04

Next, we asked whether treatment with JIB-04 alters the resistance of TN-IBC cells according to useful criteria (e.g., their sensitivity to chemotherapeutic drugs) even though they are not killed during in vitro treatment. To answer this question, we pretreated SUM149-MA cells with JIB-04, allowed them to recover and grow in the absence of JIB-04, and then tested their sensitivity to treatment with doxorubicin or paclitaxel as compared with that of control MA cells not pretreated with JIB-04. Figure 2 shows representative results of pretreatment of SUM149-MA cells with 125 nM JIB-04 for 10 days followed by recovery and growth for 7 days. We then evaluated their relative resistance to chemotherapeutic drugs, which involved 8-day treatment with either 5 nM paclitaxel or 100 nM doxorubicin followed by recovery and growth of resistant cells into colonies for 12 days . We observed many colonies (more than 200) of different sizes of control SUM149-MA cells not pretreated with JIB-04. In contrast, SUM149-MA cells pretreated with JIB-04 exhibited a dramatic reduction in the number of colonies after treatment with either paclitaxel or doxorubicin, with observation of only a few small colonies (fewer than five). These findings demonstrated that treatment with JIB-04 modulates the epigenetic state of resistant cancer cells toward a favorable therapy-sensitive state.

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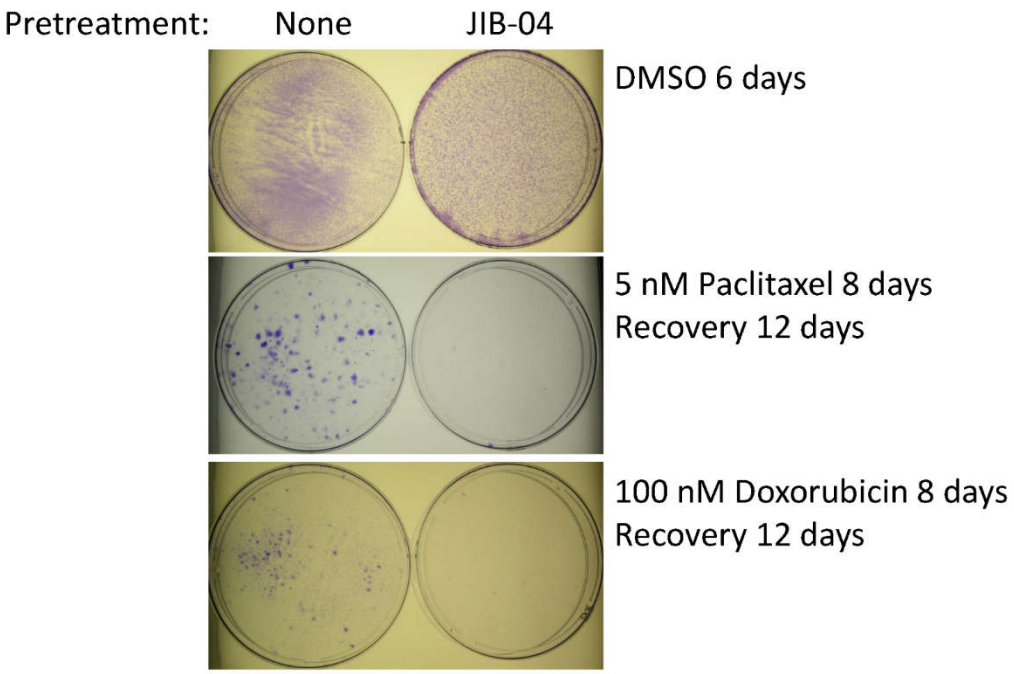

Figure 2. Treatment with JIB-04 sensitizes SUM149-MA cells to treatment with chemotherapeutic drugs. After 10-day treatment with 125 nM JIB-04, surviving cells were allowed to recover in a drug-free medium for 7 days. Following this, the cells were treated in parallel with 5 nM paclitaxel (middle panel) or 100 nM doxorubicin (lower panel) for 8 days and then allowed to recover and grow into colonies for 12 days before staining with crystal violet. Cells treated with DMSO in parallel that were stained 6 days later served as controls (top panel). Representative images of cell cultures taken at 10× magnification are shown. These images show that pretreatment with JIB-04 significantly decreased the number of colonies.

Although SUM149-MA cells are highly adaptable and resistant [9-14], we recognize that the parental cell line SUM149 is one of the most resistant TNBC cell lines. Therefore, determining whether treatment with JIB-04 also sensitizes parental SUM149 cells to chemotherapeutic drugs is useful. To that end, we pretreated SUM149-Luc cells with JIB-04 and then tested their sensitivity to paclitaxel and doxorubicin. Specifically, we pretreated the cells with 125 nM JIB-04 for 10 days, allowed them to recover and grow for 15 days, and then treated them with 5 nM paclitaxel (5 days followed by 10 days of recovery) or 100 nM doxorubicin (5 days followed by 11 days of recovery). We found that that the pretreatment resulted in dramatically fewer colonies than did control treatment with DMSO, demonstrating that the pretreatment sensitized SUM149-Luc cells to both chemotherapeutic drugs equally (Figure 3).

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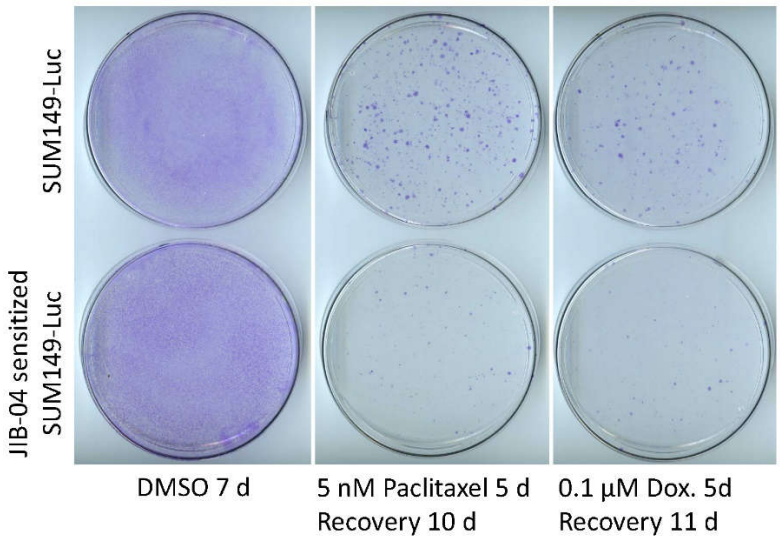

Figure 3. Pretreatment with JIB-04 sensitizes SUM149-Luc cells to chemotherapeutic drugs. After 10-day treatment with 125 nM JIB-04, surviving cells were allowed to recover in a drug-free medium for 15 days. Following this, the cells were treated in parallel with 5 nM paclitaxel (middle panel) or 100 nM doxorubicin (right panel) for 5 days and then allowed to recover and grow into colonies for 10 or 11 days as indicated before staining with crystal violet. Cells treated with DMSO in parallel and stained 7 days later served as controls (left panel). These images show that pretreatment with JIB-04 significantly decreased the number of colonies.

3.3. Increased PD-L1 expression in SUM149-MA cells upon treatment with JIB-04

Our analysis of gene expression data showed that SUM149-MA cells have reduced PD-L1 mRNA expression [14]. Considering that anti-PD-L1 antibodies are increasingly used in treatment of TNBC, we decided to validate gene expression data with Western blotting. We found that SUM149-MA cells had lower (by 60%) PD-L1 expression than did parental SUM149-Luc cells (Figure 4, left). Our interpretation of this finding is that the lower PD-L1 expression in SUM149-MA cells may result from their progenitor cell-like nature. Studies have demonstrated that TNBC cells that express PD-L1 may be more sensitive than cells not expressing it to therapies in general, not just anti-PD-L1 antibodies. A clinical trial that demonstrated markedly better pathologic complete response of PD-L1–positive TNBC than PD-L1–negative TNBC to neoadjuvant therapy [30] supports this assertion. To assess the potential clinical relevance of PD-L1 expression, we sought to determine whether treatment with JIB-04 increases PD-L1 expression in SUM149-MA cells as part of reprogramming of epigenome toward a "differentiated state" that may be a feature of relatively therapy-sensitive TNBC cells. Western blot analyses showed that 10-day treatment with 62.5 nM JIB-04 (followed by a 10-day recovery period) significantly increased (2.26-fold) the level of PD-L1 protein in SUM149-MA cells (Figure 4, right). Similar treatment with JIB-04 also significantly increased (1.94-fold) PD-L1 protein levels in parental SUM149-Luc cells (Figure 4, middle). We noted that the fold increase in PD-L1 protein level in these cells over that in untreated control cells was higher in SUM149-MA cells than in parental SUM149-Luc cells, demonstrating that resistant cells may be more sensitive to treatment with JIB-04 in this regard.

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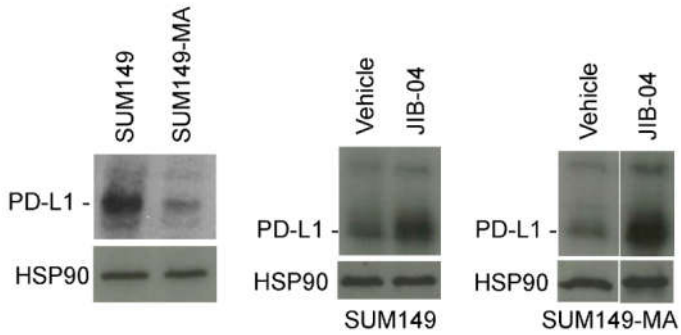

Figure 4. Increased PD-L1 protein levels in TN-IBC cells upon treatment with JIB-04. Left, the PD-L1 protein level in SUM149-MA cells was lower than that in the parental SUM149-Luc cell line. The relative levels of PD-L1 protein in SUM149-MA and parental SUM149-Luc (SUM149) cells cultured in glutamine-containing medium with dialyzed FBS were determined via Western blotting. SUM149-Luc (middle) and SUM149-MA (right) cells were treated with 62.5 nM JIB-04 for 10 days, allowed to recover for 10 days in a drug-free medium, and subjected to Western blotting to compare PD-L1 protein levels. Nitrocellulose membranes were re-probed with an anti-HSP90 antibody to normalize sample loading.

3.4. Sensitivity of FC-IBC02-MA cells to treatment with JIB-04

Next, we evaluated JIB-04 in another resistant TN-IBC cell line model that we recently developed, namely, adaptable FC-IBC02-MA cells derived from the FC-IBC02 cell line [13]. We found that 14-day treatment with 62.5 nM JIB-04 killed most of the cells in culture. However, a small number of cells survived and grew into colonies during this period. A comparison of FC-IBC02-MA and FC-IBC02 cells showed that the parental FC-IBC02 cells yielded a significant number of colonies, whereas the FC-IBC02-MA cells yielded only a few colonies (Figure 5). This significant difference in the number of colonies suggested that resistant cells have increased sensitivity to JIB-04. This result along with the results described above for SUM149-MA cells support the idea, which is also backed by several published studies on different cancers, that JIB-04 may be a suitable agent for targeting resistant cancer cells in patients with poor-prognosis cancers [20-26].

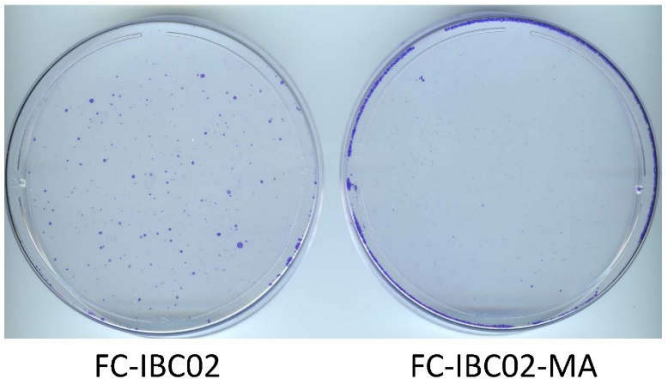

Figure 5. Inhibition of the growth of FC-IBC02 and FC-IBC02-MA cells by treatment with JIB-04. Cultures of both cell lines were treated in parallel with 62.5 nM JIB-04 for 14 days and then stained with crystal violet. Based on a comparison of the number of colonies in the images, JIB-04 affected the FC-IBC02-MA cells much more severely than it did the parental FC-IBC02 cells.

4. Discussion

Using a novel cell culture model of deep intrinsic treatment resistance of cancer, which allows for therapeutic evaluation in rare cancer cells that can persist in reversible quiescence under a variety of pressures, we showed that treatment with low-dose JIB-04 may inhibit progression of breast cancer. Concerning how treatment with JIB-04 may sensitize resistant cancer cells, modulation of the epigenome may shift cell fate from stem-like to non-stem-like. However, we must remember that analogous to the persistence of a

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pool of stem-like cancer cells in the body, such a pool also likely persists in our system under treatment with JIB-04. We hypothesize that although treatment with JIB-04 does not completely eradicate resistant cancer cells, it does change them enough that it may provide a clinical benefit. Our results demonstrated that JIB-04 may go deeper at intrinsic resistance than other therapies that mainly target proliferative cancer cells.

Although we did not investigate which specific histone demethylases are the targets of JIB-04 in our model, two specific Jumonji histone demethylases are of interest. Specifically, our previously published gene expression and gene copy number data demonstrated that JMJD1B/KDM3B was amplified and JHDM1D/KDM7A was overexpressed in SUM149-MA cells [14]. Of note is that both of these demethylases were also overexpressed in paclitaxel- and carboplatin-resistant lung cancers and that resistant cells were hypersensitive to JIB-04 treatment in preclinical models of lung cancer [22]. These results raise the possibility that KDM3B and KDM7A are targets of JIB-04 in resistant TNBCs such as TN-IBC, which can be investigated in future.

If validated further in preclinical animal studies, epigenetic modulators like JIB-04 may be suitable for evaluation in clinical trials to determine their potential to inhibit relapse in high-risk patients such as those with TN-IBC. As to how JIB-04 could be useful in the setting of immune checkpoint blockade therapy for TNBC, it is worth mentioning that in some studies anti-PD-L1 antibodies were offered to TNBC patients based on their PD-L1 positivity, as it increases the likelihood of response [31]. Other studies have not shown response dependent on PD-L1, possibly due to use of different drugs, different disease stages (early versus late), and/or PD-L1 assays utilized [32]. In any case, if treatment with JIB-04 alters TNBC cells similarly to what we observed in cell culture (i.e., increases their PD-L1 expression), a useful next step would be to determine whether pretreatment with JIB-04 converts the PD-L1 status from negative to positive in mouse models and patients with high-risk TNBC, thus converting anti-PD-L1 therapy non-responders to responders.

5. Conclusions

Based on our results described herein, we concluded that JIB-04 is a suitable agent for overcoming therapy resistance of cancers such as TN-IBC. We obtained our results using a cell culture model of deep intrinsic resistance. Others have reported similar findings for several cancer types using different experimental models, specifically, cell lines and xenograft tumor models in mice. Whether JIB-04 or compounds similar to it will advance to clinical trials remains to be seen.

Besides providing useful information regarding the potential of JIB-04 to overcome therapy resistance IBC, this study further validates our cell culture-based model of deep intrinsic resistance as a useful addition to other models that guide development of cancer therapy. Application of this model with various cancers has the potential to expedite therapy development and thus improve outcomes.

Supplementary Materials: Figure S1: Uncropped Western blots used for Figure 4.

Author Contributions: B.S. designed the study, performed data analysis, prepared figures, and wrote the manuscript. V.N.S. performed experiments and data analysis. A.L. supervised the study, provided intellectual input, and acquired funding. All authors reviewed and revised the manuscript and agreed to publication of it.

Funding: This study was supported by a grant from the State of Texas Rare and Aggressive Breast Cancer Research Program, by the Breast Cancer Metastasis Research Program at MD Anderson, and by the NIH/NCI under award number P30CA016672 and used the Cytogenetics and Cell Authentication Core.

Acknowledgments: We thank Laura J. Washburn, Hannah E. Kinne, and Ryan D. Milligan for conducting some of the experiments. We thank members of the MD Anderson Morgan Welch Inflammatory Breast Cancer Research Program and Clinic for discussions and comments during the study. We thank Donald R. Norwood of MD Anderson Editing Services, Research Medical Library for editorial help.

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Conflicts of Interest: None of the authors declare any conflicts of interest.

Abbreviations: 6-MP: 6-mercaptopurine; DMSO: dimethyl sulfoxide; FBS: fetal bovine serum; IBC: inflammatory breast cancer; TNBC: triple-negative breast cancer; TN-IBC: triple-negative inflammatory breast cancer; MA: metabolically adaptable.

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