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Report

Ineffectiveness of doxorubicin treatment on solitary dormant mammary carcinoma cells or late-developing metastases

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Summary

Breast cancer is noted for long periods of tumor dormancy and metastases can occur many years after treatment. Adjuvant chemotherapy is used to prevent metastatic recurrence but is not always successful. As a model for studying mechanisms of dormancy, we have used two murine mammary carcinoma cell lines: D2.0R/R cells, which are poorly metastatic but form metastases in some mice after long latency times, and D2A1/R cells, which form more numerous metastases much earlier. Previously we identified a surprisingly large population of dormant but viable solitary cells, which persisted in an undivided state for up to 11 weeks after injection of D2.0R/R cells. Dormant cells were also detected for D2A1/R cells, in a background of growing metastases. Here we used this model to test the hypothesis that dormant tumor cells would not be killed by cytotoxic chemotherapy that targets actively dividing cells, and that the late development of metastases from D2.0R/R cells would not be inhibited by chemotherapy that effectively inhibited D2A1/R metastases. We injected mice with D2A1/R or D2.0R/R cells via a mesenteric vein to target liver. We developed a doxorubicin (DXR) treatment protocol that effectively reduced the metastatic tumor burden from D2A1/R cells at 3 weeks. However, this treatment did not reduce the numbers of solitary dormant cells in mice injected with either D2A1/R or D2.0R/R cells. Furthermore, DXR did not reduce the metastatic tumor burden after an 11-week latency period in mice injected with D2.0R/R cells. Thus, apparently effective chemotherapy may spare non-dividing cancer cells, and these cells may give rise to metastases at a later date. This study has important clinical implications for patients being treated with cytotoxic chemotherapy.

Introduction

The presence of undetected micrometastases, disseminated before or around the time women receive local treatment for 'early' breast cancer, is assumed to be the reason why many subsequently develop overt distant metastases ultimately causing death. Adjuvant chemotherapy can reduce recurrence rates and improve survival for some women [1], with greater benefit shown for anthracycline (e.g., doxorubicin [DXR]) based polychemotherapy. Such chemotherapy is not effective in all women, however [2], and reasons for failure may include a large initial tumor burden, poor drug access to tumor cells and primary drug resistance [3]. Here we present *in vivo* results to support a novel mechanism that might explain some cancer chemotherapy failures.

Many chemotherapeutic agents, including DXR, preferentially target actively dividing cells [4, 5]. Clinically, tumors with more rapid doubling times tend to be the most chemosensitive, and less responsive tumors tend to have slower doubling times [6, 7]. Similarly, normal tissues that are rapidly cycling, such as the bone marrow, alimentary mucosa, and hair follicles, tend to be sensitive to cytotoxic chemotherapy, leading to many of the side-effects of chemotherapy, including myelosuppression, nausea and vomiting, and alopecia [6, 7]. It is thus likely that

many disseminated cancer cells that were not dividing would not be killed by cytotoxic chemotherapy, and if these cells were present in large numbers, they could be responsible for cancer recurrence and development of metastases at a later date.

Studies on the biology and responsiveness to treatment of dormant cancer cells have been limited by the lack of suitable animal models in which disseminated, dormant cells can be detected and quantified. We have recently provided experimental evidence for the presence of a surprisingly large population of solitary dormant cancer cells following injection of cells to target mouse liver, both for D2A1/R mammary carcinoma cells, existing in a background of rapidly growing liver metastases, as well as for poorly metastatic D2.0R/R mammary carcinoma cells [8]. These solitary cells were dormant, as defined by both retention of an exogenous fluorescent marker that is lost with cell division, and lack of staining with the proliferation marker Ki67. At least some of these cells were shown to be viable, and were able to grow *in vitro* in cell culture and *in vivo* after re-injection into a mammary fat pad. These cells thus represent an experimental *in vivo* dormancy model, where solitary dormant cells may persist in the absence, or even the presence, of rapidly growing metastases [8].

Here we used the same animal model to test the hypothesis that the numbers of dormant D2A1/R or D2.0R/R mammary carcinoma cells would not be reduced by a DXR chemotherapy protocol that effectively reduced metastatic burden from D2A1/R cells, and that late-developing metastases from D2.0R/R cells would not be inhibited.

Materials and methods

Cell lines and fluorescent cell labeling

D2A1/R and D2.0R/R murine mammary carcinoma cell lines (D2A1 and D2.0R cells transfected with the pEGFP-C2 vector) [8, 9] were maintained in tissue culture (37°C, 5% CO_2 humidified atmosphere) in Dulbecco's modified Eagle's medium (DMEM; Canadian Life Technologies Inc., Burlington, ON) supplemented with 10% fetal calf serum (FCS; Canadian Life Technologies Inc.). Prior to injection, cells were labeled with fluorescent nanospheres, as employed earlier for *in vivo* detection of cancer cells that had not undergone cell division since their injection into the animal [8, 9]; although the cells had been previously transfected with EGFP, this label did not provide sufficient fluorescence alone to detect solitary cells, necessitating the use of the additional label.

Experimental metastasis assays and DXR treatment

Female severe combined immunodeficient (SCID) mice, 6–7 weeks of age (Charles River, St. Constant, Quebec, Canada), were cared for in accordance with the standards of the Canadian Council on Animal Care, under an approved protocol of the University of Western Ontario Council on Animal Care. Mice were anesthetized using ketamine/xylazine mixture (1.6 mg ketamine and 0.08 mg xylazine/15 g body mass) administered by i.p. injection, as described [8]. Fluorescent nanosphere-labeled D2A1/R or D2.0R/R cells (3×10^5 cells/mouse) were co-injected with 10.2 ± 0.1 μm microspheres (at a ratio of 5 cells:1 microsphere) via a mesenteric vein to target the cells to the liver, as described [8, 10–12]. The microspheres provide a reference for the number of cells that are originally delivered to a tissue volume, and permit cell survival at later times to be quantified [8, 10–12]. Buprenorphine analgesic (0.1 mg/kg body weight) was administered s.c. as mice awoke and 24 h after surgery [8].

Eight days after surgery, mice injected with each cell line were randomized into treatment and control groups and treated either with DXR (1 mg/kg) or PBS (0.2 ml) as a control. DXR or PBS was administered i.p., every second day, for a total of 6 times, at days 8, 10, 12, 14, 16, and 18 after surgery, as diagrammed in Figure 1. Preliminary *in vivo* experiments, based on published studies [13, 14], had identified this dose

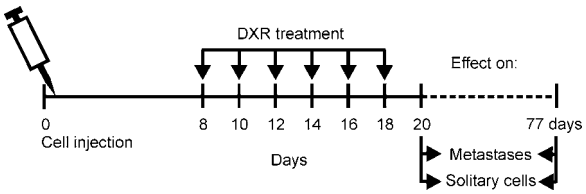

Figure 1. Experimental metastasis assay and DXR treatment regimen. A suspension of fluorescently labeled D2A1/R or D2.0R/R cells and 'accounting' microspheres was injected via a mesenteric vein to target them to mouse liver. Mice were randomized into treatment groups ($n = 8$–10 mice per group, as indicated in the text) and treated either with DXR or PBS control, starting at day 8 after cell injection. Treatments were administered i.p., once every second day, for a total of six times. Mice injected with D2A1/R cells were sacrificed at day 20 due to significant tumor burden. Mice injected with D2.0R/R cells were sacrificed at either day 20 or day 77. The effect of DXR treatment on metastasis growth and dormant solitary cell survival was then assessed.

of DXR as effective and with minimal toxicity to the mice, while a higher dose (2 mg/kg) was no more effective but was associated with unacceptable toxicity (data not shown). Preliminary *in vitro* experiments demonstrated that both D2A1/R and D2.0R/R cells were equally and highly sensitive to DXR under tissue culture conditions where the cells were actively dividing (data not shown).

Mice were sacrificed 20 or 77 days after surgery and livers were immediately removed and fixed in 10% neutral buffered formalin (pH 7.6). The livers were then examined for visible surface tumors. For both cell lines, the effect of DXR on two distinct populations of cells in secondary sites was then assessed: dormant solitary cells versus cells in actively growing metastases. To do this, representative sections of the fixed livers were either cut in 50 μm-thick sections (for quantitative assessment of solitary cell survival using the cell 'accounting' technique) or were embedded in paraffin (for histopathological quantification of metastatic tumor burden).

Cell 'accounting' in tissues to quantify solitary cells

To determine the proportions of the injected cancer cells that persist in the tissue as solitary cells, it is necessary to express the number of cells observed in a tissue sample at the end point of the experiment, relative to the number of cells originally entering that volume. The cell 'accounting' technique was developed for this purpose and has been previously used to quantify dormant D2A1 and D2.0R cells, as defined by retention of nanosphere fluorescence and corresponding to negative staining for Ki67 and TUNEL by immunohistochemistry [8, 9]. The fixed livers were sectioned (∼50-μm thick) using a vibratome, as described previously [8, 11]. At least three representative sections were obtained from each liver, amounting to a total of at least 24 sections per treatment group/per cell line. These thick sections were analyzed for number of solitary, nanosphere-retaining cancer cells and accounting microspheres using an inverted microscope equipped with epifluorescence illumination (excitation wavelength 450–490 nm). Epillumination through the microscope objective was used to excite fluorescently labeled cells and accounting microspheres. Digital images of representative views of cancer cells and microspheres were captured using a video camera and saved on a computer. Tissue adjacent to the thick sections was embedded in paraffin for tumor burden assessment.

Tumor burden assessment

Percent metastatic burden was calculated based on quantitative analysis of tumor-to-normal tissue ratios in H&E stained liver sections. Digital images of the sections were acquired and analyzed, as described previously [15]. Percent tumor burden (tumor area/total area) was calculated based on at least five sections for each mouse (i.e., 40–50 sections per treatment group).

Statistical analysis

Data are expressed as the mean ± SE. Statistical analyses were performed using Sigma Stat version 2.03 for Windows (Access Softek Inc., San Rafael, CA). To assess the relationship between specific factors, a Student's *t*-test or Mann–Whitney rank sum test was performed, as appropriate. A level of $P < 0.05$ was regarded as statistically significant.

Results

Effect of DXR on D2A1/R metastases and solitary cells

DXR was effective at significantly reducing the metastatic burden in mice injected with D2A1/R cells, at the end point of 20 days after injection. At this time, metastases occupied $37 \pm 6.5\%$ ($n = 8$ mice) of the total liver volume in control PBS-treated animals versus $9 \pm 3.2\%$ ($n = 10$) in DXR-treated mice (Figure 2(A)), as illustrated in Figure 3(A and B). The difference in metastatic burden between these two treatment groups was highly significant ($P < 0.001$). Thus, this DXR treatment led to a four-fold reduction in D2A1/R liver metastases.

We then determined the effect of DXR treatment on persistence of solitary dormant D2A1/R cells in the livers of the same mice, using the reference microsphere cell 'accounting' technique (8, 12–14). The control and DXR treatment groups showed similar ($P > 0.9$) D2A1/R solitary cell survival: $17.6 \pm 2.9\%$ ($n = 8$ mice) versus $17.5 \pm 2.5\%$ ($n = 10$) of the cells originally injected (Figures 2(B) and 3(C and D)). These values are comparable to those quantified previously for D2A1/R cells at this time point [8]. Thus, DXR treatment had no significant effect on the survival of D2A1/R solitary dormant cells *in vivo*, al-

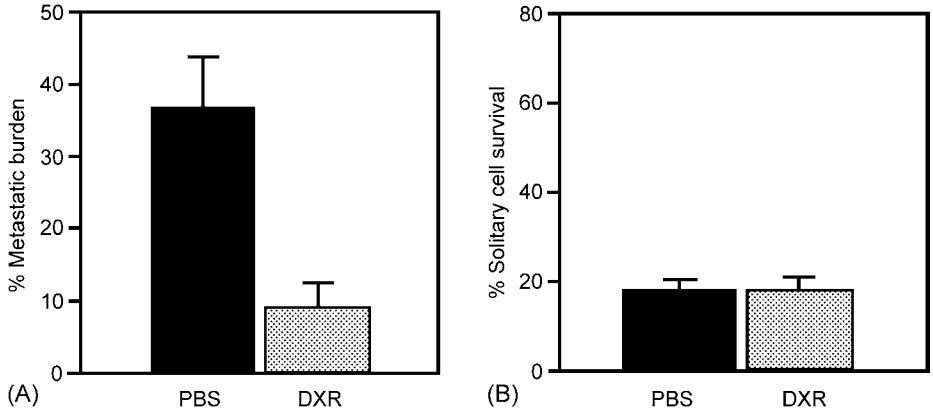

Figure 2. Effect of DXR treatment on metastases and dormant solitary cells in mice injected with D2A1/R cells. (A) DXR treatment inhibited metastasis in D2A1/R-injected mice. Percent metastatic burden was calculated based on quantitative analysis of tumor-to-normal tissue ratios in H&E stained liver sections. PBS-treated mice had significantly higher volume ($37 \pm 6.5\%$ of the total liver volume) occupied by metastases, when compared to DXR-treated mice ($9 \pm 3.2\%$)($P < 0.001$). (B) DXR treatment had no significant effect on the survival of D2A1/R solitary dormant cells. DXR-treated mice showed similar solitary cell survival, compared to PBS-treated (control) mice ($P > 0.9$). Solitary cell survival was quantified by counting the number of microspheres in 50-μm thick tissue sections to estimate the number of cells originally arrested and comparing this value with the number of cells observed in the same tissue volume, as described previously [8, 10–12]. Percent survival is comparable to previous results with these cells [8]. Bars represent mean percent solitary cell survival, ±standard error.

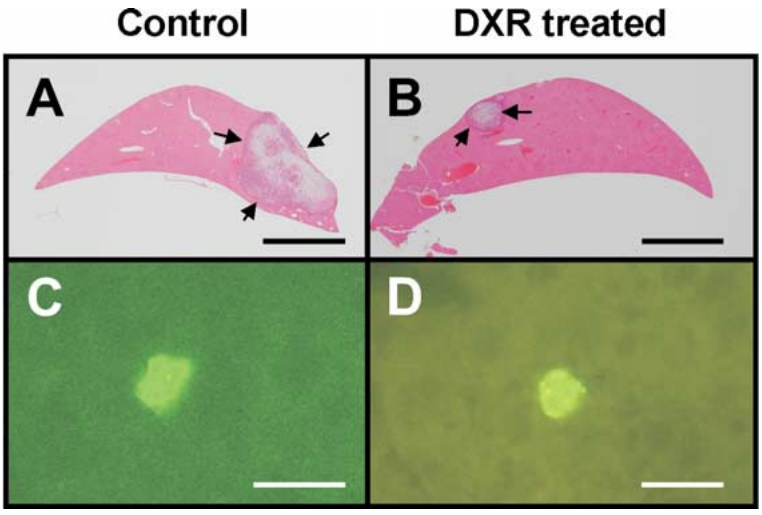

Figure 3. DXR inhibition of D2A1/R liver metastases and lack of effect on dormant solitary cells. (A and B) Images of representative H&E stained 4 μm -thick liver sections containing D2A1/R metastases (arrows) after PBS (control) or DXR treatment. DXR treatment led to reduction in metastatic tumor burden, when compared to control-treated animals. For PBS-treated animals, metastases occupied approximately 40% of the total liver volume versus ∼10% in DXR-treated mice. Images represent the mean tumor burden values graphed on Figure 2(A). Bars = 3 mm. (C and D) Images of representative nanosphere-labeled D2A1/R dormant solitary cells as detected in 50-μm thick liver sections after PBS (control) or DXR treatment. Although DXR treatment substantially reduced metastatic burden, it had no significant effect on the survival of D2A1.R solitary dormant cells in the same organs. Bars = 20 μm (C) and 25 μm (D).

though the DXR treatment substantially reduced metastatic burden from D2A1/R cells in the same organs.

Effect of DXR on D2.0R/R solitary cells and late-forming metastases

We assessed the effect of DXR treatment on persistence of solitary D2.0R/R cells at two time points, 20 and 77 days after injection of the cells. At 20 days, DXR treatment did not affect the numbers of solitary cells detected in mice injected with poorly metastatic D2.0R/R cells and there was no difference in numbers of solitary D2.0R/R cells detected in livers of mice treated with DXR versus PBS. We found that $57.6 \pm 6.6\%$ ($n = 10$ mice) of the injected D2.0R/R cells remained as undivided fluorescent cells in the

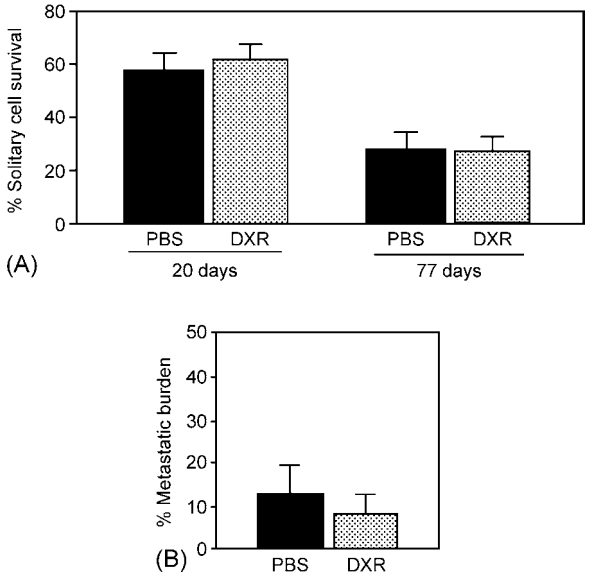

Figure 4. Effect of DXR treatment on dormant solitary cells and late-forming metastases in mice injected with D2.0R/R cells. (A) DXR-treatment had no effect on the survival of solitary D2.0R/R cells at either 20 or 77 days. There was no significant difference in solitary cell survival between the two treatment groups ($P = 0.66$ for 20 days; $P = 0.93$ for 77 days). Percent survival at 20 days is comparable to previously reported results with these cells [8]. Bars represent mean percent solitary cell survival, ±standard error. (B) DXR-treatment had no effect on the metastatic burden from late-forming metastases detected at 77 days. There was no significant difference in metastatic tumor burden between the two treatment groups ($P = 0.72$). Bars represent mean percent tumor burden, ±standard error.

PBS-treated mice, versus $61.5 \pm 5.9\%$ ($n = 10$) in the DXR-treated mice (Figure 4(A)) ($P = 0.66$). These values are comparable to those quantified previously for D2.0R/R cells at this time point [8]. Similarly, at 77 days there was no difference in the numbers of solitary D2.0R/R cells detected in livers of mice treated with DXR versus PBS. We found that $27.4 \pm 12.5\%$ ($n = 8$ mice) of the injected D2.0R/R cells remained as undivided fluorescent cells in the PBS-treated mice, versus $28.0 \pm 15.6\%$ ($n = 8$) in the DXR-treated mice (Figure 4(A)) ($P = 0.93$).

The DXR treatment also had no effect on the late-developing metastases in mice injected with D2.0R/R cells. At 77 days, D2.0R/R metastases occupied $13.4 \pm 6.5\%$ ($n = 8$ mice) of the total liver volume in control PBS-treated animals versus $8.7 \pm 4.4\%$ ($n = 8$) in DXR-treated mice (Figure 4(B)). No statistical difference between the tumor burdens of these two groups was found ($P = 0.72$). Thus, the same DXR treatment that successfully inhibited metastases formed by the highly metastatic D2A1/R cell line had no significant effect on either the survival of single dormant D2.0R/R cells at 20 or 77 days, or the late-forming metastases formed by 77 days by these cells after a period of dormancy.

Discussion

In this study we asked whether cytotoxic chemotherapy could reduce the numbers of dormant cancer cells present in a secondary site. We hypothesized that solitary dormant cells present in the tissue would be spared by cytotoxic chemotherapy that effectively reduces metastatic burden, and that such cells could then give rise to metastases at a later time. To address this question, we used a pair of murine mammary carcinoma cell lines that we have previously characterized [8, 9]: D2.0R/R cells which are poorly metastatic and form metastases in some mice after long latency times, and more highly metastatic D2A1/R cells. Both cell lines were equally sensitive to DXR *in vitro*, under conditions where they would be actively dividing. Using D2A1/R cells we could test whether DXR would affect dormant cells at a time when the drug was effective against actively growing metastases, but because the early D2A1/R metastases are generally lethal beyond 3 weeks, the long-term metastatic potential of the surviving dormant cells could not be assessed. However, we had previously found that mice survive for at least 11 weeks with large numbers of D2.0R/R cells persisting in secondary sites as dormant, solitary but viable cells [8]. Thus, we could employ the same experimental protocol using D2.0R/R cells to assess the effects of early DXR treatment on the long-term metastatic potential of dormant cells.

Here we first devised a DXR treatment regimen that successfully inhibited metastases formed by the highly metastatic D2A1/R cell line. We found that the numbers of dormant D2A1/R cancer cells were not affected by this treatment, in spite of the success in treating actively growing metastases in the same organs of these mice. Nor were numbers of solitary cells in mice injected with poorly metastatic D2.0R/R cells affected by the same DXR-treatment protocol used for the highly metastatic cells. This DXR-treatment protocol also failed to inhibit the development of late-forming metastases in mice injected with D2.0R/R cells.

It has been assumed that cytotoxic chemotherapy will preferentially target cells that are actively dividing, and would be ineffective against those cells that are not dividing [4, 5]. This study made use of

a murine model of mammary carcinoma dormancy that allowed this hypothesis to be directly tested *in vivo*, and showed that DXR chemotherapy that was effective against actively growing metastases did not reduce numbers of dormant solitary cancer cells in a secondary site. Further, it was demonstrated that the subsequent ability of these dormant cells to form metastases was not impaired by previous DXR treatment.

Metastases can occur years or even decades after apparently successful primary treatment of breast and other cancers [16–18]. Thus, tumor dormancy and the inability to predict with certainty which patients will subsequently develop metastases, makes treatment decisions difficult and introduces years of uncertainty for many patients. Excess mortality, extending for at least 20 years, was described in several early studies evaluating the curability of breast cancer [19–22]. Detailed information on the time course of recurrences after mastectomy has been documented in two large series of women who did not receive systemic adjuvant therapy [23, 24]. In both studies, the authors felt their findings were consistent with a tumor dormancy hypothesis. Similarly, Louwman et al. [25], in a population-based study of breast cancer patients, found that in comparison with a normal female population, the mortality ratio for breast cancer was substantially elevated even after 20 years. Late recurrences also were documented in a large series of women receiving adjuvant chemotherapy, continuing through to year 12 [26]. In women receiving adjuvant chemotherapy after surgery for node positive breast cancer, Gasparini et al. [27] observed a late peak of incidence of metastasis at 60 months, which was related to intratumoral microvascular density. Their postulated causes included changes in factors such as cell adhesion, tumor cell motility, metastasis-suppressing genes and immune response. Equally, based on our findings, subsequent growth of a population of dormant cells unresponsive to chemotherapy may lead to a later peak in recurrence.

Mathematical modeling based on survival data from women with breast cancer [28, 29] supports the concept that tumor cells may exist in various distinct states during periods of clinical tumor dormancy, some of which might render the cells less sensitive to chemotherapy that targets actively dividing cells. We have identified the persistence of large numbers of solitary, dormant cells, in secondary sites in several experimental metastasis models, including murine mammary carcinoma cells [8], ras-transformed NIH 3T3 cells [15], and B16F1 [11] and B16F10 [12] melanoma cells. We have hypothesized that such cells would be unaffected by cytotoxic chemotherapy [30], and this hypothesis was directly tested in the present study. Other experimental models have identified 'dormant', pre-angiogenic micrometastases, in which proliferation is balanced by apoptosis, resulting in no net growth, as a possible contributor to tumor dormancy [31, 32]. In contrast to solitary dormant cancer cells, cells in 'dormant' pre-angiogenic micrometastases might be expected to be relatively sensitive to cytotoxic chemotherapy, in that they are actively dividing.

Several studies have identified both isolated solitary cancer cells and micrometastases in cancer patients' blood and tissues, using a variety of techniques [33–41]. However, the clinical implications of such cells remain uncertain, as their presence does not always correlate with poor prognosis. In part this lack of prognostic value may be due to lack of standardization of the evolving methodologies for detection, as well as to the need for markers to predict the future metastatic potential of subsets of cells. Additionally, solitary cells and micrometastases may be biologically distinct populations, which may differ in their prognostic value, and thus perhaps should be examined separately.

The experimental findings presented here indicate that the number, viability and subsequent metastatic ability of dormant cancer cells may not be reduced by chemotherapy that is effective against actively growing metastases. This finding has important clinical implications as it provides *in vivo* evidence for a mechanism that could be responsible for some of the failures of cancer chemotherapy. For patients undergoing adjuvant chemotherapy, it is possible that dormant cells will be unaffected by treatment, even if the cells are inherently drug-sensitive when they are in an actively dividing state. Interestingly, the results presented here are consistent with a recent clinical study that found that chemotherapy in a group of high-risk breast cancer patients did not reduce the presence of solitary tumor cells in bone marrow [42]. Solitary dormant cancer cells may retain the potential to begin growth at a later date, thus leading to an uncertainty in the prognosis for patients treated in the adjuvant setting. The findings presented here point to the need for better understanding of the biology of solitary dormant cells, to identify conditions that can lead to their activation, to determine if there are markers that can predict high versus low probability of this activation in patients [43], and to discover ways to eliminate these cells.

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