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Effects of the MCP-1 synthesis inhibitor bindarit on tumorigenesis and inflammatory markers in the C3(1)/SV40Tag mouse model of breast cancer

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Abstract

Breast cancer, the most deadly cancer in women, is characterized by elevated levels of inflammation within and surrounding the tumor that can lead to accelerated growth, invasion and metastasis. Macrophages are central to the inflammatory milieu and are recruited to the tumor microenvironment by several factors including monocyte chemoattractant protein-1 (MCP-1). Using the anti-inflammatory molecule bindarit to target MCP-1, we investigated the role of this chemokine on macrophage related inflammation and mammary tumorigenesis in a transgenic mouse model of breast cancer. C3(1)/SV40Tag mice and wild type FVB/N were randomized to either control or 0.5% bindarit diet from 4–21 weeks of age. Tumor number and volume were recorded over time and at sacrifice. Macrophage markers as well as inflammatory mediators were examined in the tumor tissue and mammary glands. Circulating MCP-1 and IL-6 were measured by ELISA. Bindarit treatment reduced tumor number ($P<0.05$), but did not affect tumor size, tumor weight or tumor latency in C3(1)/SV40Tag mice. Within the tumor, mRNA expression of bindarit's primary targets, MCP-1 and IL-12/p35, were significantly decreased by bindarit treatment ($P<0.05$), and this was consistent with trends for reduced expression of TNF- α , IL-6, F4/80, CD206, and IL-10. In mammary tissue, expression of MCP-1, TNF- α , IL-6, F4/80, IL-10 and IL-12/p35 was significantly elevated in C3(1)/SV40Tag mice compared to wild type FVB/N mice, but IL-6 was the only marker decreased by bindarit treatment ($P<0.05$). Plasma MCP-1 was highly correlated with tumor volume ($P<0.05$); however, it was not affected by bindarit at 21 weeks of age. Similarly, circulating IL-6 was increased in C3(1)/SV40Tag mice but there was no effect of bindarit treatment. These results show that tumor multiplicity in the C3(1)/SV40Tag mouse model of breast cancer is reduced by bindarit, however these effects are independent of

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changes in plasma levels of MCP-1 and IL-6, but may be related to the attenuated expression of MCP-1 along with several inflammatory mediators and macrophage markers within the tumor.

Keywords

C3(1)/SV40Tag transgenic mice; bindarit; mammary cancer; MCP-1

1. INTRODUCTION

Breast cancer is currently the second most commonly diagnosed cancer and the leading cause of cancer related death in women in the United States [1]. Inflammation plays a significant role as a predisposing event, as well as in the promotion and progression of the disease. Macrophages are central to the inflammatory response in breast cancer; tumor associated macrophages (TAMs) contribute to tumor onset and development through the promotion of chronic inflammation, tumor cell invasion, angiogenesis and metastasis [2, 3]. In breast carcinomas, TAMs can account for more than 50% of the tumor mass and high numbers of TAMs have been correlated with poor patient prognosis in 80% of tumor cases [4, 5]. Further, a high macrophage density in breast carcinoma samples was reported to be a significant independent prognostic indicator of both relapse free survival and overall survival [2, 5, 6]. Therefore, identifying strategies to minimize the negative impact of TAMs in breast cancer is of importance.

Monocyte chemoattractant protein-1 (MCP-1) is significantly correlated with TAM accumulation in primary breast tumors [7]. In fact, MCP-1 has been suggested to be the most important chemokine for macrophage recruitment to the tumor microenvironment [8]. MCP-1 carries significant prognostic value for relapse free survival, is correlated with high tumor grade and lymph node metastasis, and is associated with low levels of differentiation and poor prognosis in breast cancer patients [7, 9–13]. In contrast to normal breast epithelial cells which lack MCP-1 expression, high levels of MCP-1 have been observed in both invasive and non-invasive ductal carcinomas leading to the belief that MCP-1 expression may be an important aspect of tumorigenesis gained during the malignant process [14]. Accordingly, treatment of a breast cancer mouse implantation model with an MCP-1 antibody decreased macrophage number, tumor angiogenesis and volume [15]. Based on these findings MCP-1 represents a potential therapeutic target for breast cancer treatment.

Bindarit [2-((1-benzyl-indazol-3-yl) methoxy)-2-methyl propionic acid] is a well characterized small synthetic indazolic derivative best known for its transcriptional inhibition of the monocyte chemoattractant subfamily of CC chemokines including MCP-1/CCL2 [16]. Treatment of carcinomas with bindarit was first performed in a human melanoma mouse model in which twice daily injections reduced tumor volume, abolished tumor macrophage recruitment and eliminated tumor vascularization [17]. More recently, bindarit significantly decreased macrophage infiltration and local tumorigenesis in syngenic Balb/c mice injected with murine breast cancer cells [18]. However, the long-term effects of bindarit treatment in a transgenic mouse model of breast cancer have not yet been determined.

The C3(1)/SV40Tag mouse model of breast cancer exists on a FVB/N background and is representative of the human disease; lesions that develop by 8–12 weeks of age are histologically similar to mammary intraepithelial neoplasia (MIN) and ductal carcinoma in situ (DCIS) observed in humans [19, 20]. Mammary tumors develop with a 100% incidence in transgenic female mice and progress to invasive carcinomas at ~16 weeks of age making this a timely and appropriate model for treatment studies [19, 20]. Previously, we have shown significant elevations in plasma MCP-1 in C3(1)/SV40Tag mice compared to FVB/N control mice as well as a significant correlation between plasma MCP-1 and tumor volume [21].

The purpose of the present investigation was to characterize the role of MCP-1 on mammary tumorigenesis in the triple negative C3(1)/SV40Tag transgenic mouse model of breast cancer by using bindarit to attenuate MCP-1 expression. We hypothesized that treatment with bindarit would significantly reduce gene expression and protein concentration of MCP-1 in the tumor microenvironment and that this would be associated with a decrease in tumor macrophage expression. Furthermore, we expected that these changes would be associated with a decrease in tumorigenesis.

2. METHODS

2.1 Animals

Female FVB/N mice were purchased from Harlan Sprague-Dawley Laboratories and bred with male heterozygous C3(1)/SV40Tag mice (a gift from Dr. Jeffrey Green, Chief, Transgenic Oncogenesis and Genomics Section, Laboratory of Cancer Biology and Genetics, National Cancer Institute) in the animal research facility at the University of South Carolina. Female offspring were genotyped by tail snips at 3 weeks old. Mice were maintained on a 12:12h light-dark cycle in a low-stress environment (22°C, 50% humidity and low noise) and provided with food and water *ad libitum*. All animal experimentation was approved by the University of South Carolina's Institutional Animal Care and Use Committee.

2.2 Treatment

Following weaning at 4 weeks of age, C3(1)/SV40Tag mice on an FVB/N background (n=14–15/gr) and wild-type FVB/N mice (n=10/gr) were randomized to either bindarit (Bin) or placebo (Con) treatment (FVB-Con, n=10; FVB-Bin, n=10; C3-Con, n=15; C3-Bin, n=14). Bindarit synthesized by Angelini (Aziende Chimiche Riunite Angelini Francesco [ACRAF], Italy) was incorporated into the AIN-76A pellet diet (BioServ, Frenchtown, NJ) at a dose of 0.5% and fed to the mice from 4–21 weeks of age [22, 23]. This dose has been used and tested in mice and was found to achieve consistent plasma bindarit levels of ~140ug/ml in female mice which corresponds to ~400uM, and is within the range reported to effectively inhibit MCP-1 in vitro (Product data sheet, Angelini Research Center) [16, 22, 23]. Further, this level of bindarit is comparable to levels achieved after the administration of 100mg/kg via oral gavage (personal communications with Angelini Research). Incorporation of bindarit into the diet was determined to be the preferable mode of administration as it provided the least invasive and least stressful method for consistent drug

administration during this long-term experiment. While no adverse side effects have been reported with bindarit treatment, all mice were closely monitored for signs of toxicity or poor health throughout the experimental period. The control groups consumed the AIN-76A pellet diet from 4–21 weeks of age. Body weight as well as food and water intake were measured weekly throughout the treatment period.

2.3 Tumor Progression

Beginning at 10 weeks of age, all C3(1)/SV40Tag mice were examined twice a week for palpable tumors by the same trained investigator. C3(1)/SV40Tag mice typically develop palpable mammary tumors between 12 and 16 weeks of age [19, 24]. Upon palpitation of a tumor, calipers were used to measure the longest and shortest diameter of the tumor. The number of tumors within each mouse was recorded and the tumor volume was calculated for each using the formula: $0.52 \times (\text{largest diameter}) \times (\text{smallest diameter})^2$, as previously described [25].

2.4 Sacrifice and Tissue Collection

At 21 weeks of age all mice were sacrificed via isoflurane inhalation. Visible tumors were dissected from all ten mammary glands and measured to determine tumor weight and tumor volume as described above. All remaining thoracic mammary gland tissue was then removed from both the right and left side. This tissue was either snap frozen in liquid nitrogen for gene expression analysis or fixed in 10% neutral buffered formalin for 24hrs (Fisher Scientific, Pittsburg, PA) for immunohistochemical analysis. Spleen weight was recorded as it has been positively correlated with tumorigenesis [21].

2.5 Immunohistochemistry

Mammary gland sections were processed to visualize any histopathological effects of the treatment. Formalin-fixed, paraffin-embedded sections were deparaffinized in xylenes and rehydrated in graded alcohol washes. H&E staining was then performed. Images were taken using the DAKO Chromavision Systems ACIS 3 system.

2.6 Plasma MCP-1 and IL-6

At sacrifice blood was collected from the inferior vena cava. Plasma was isolated after centrifugation (10 minutes at 10,000g) and was then stored at -80°C until analysis. Plasma levels of MCP-1 and IL-6 were measured using ELISA techniques (R&D Systems, Minneapolis MN) according to the manufacturer's instructions. All samples were run in duplicate when sample volume permitted.

2.7 Concentration of inflammatory markers

Mammary tumor tissue was homogenized in iscoves protein medium using a polytron and samples were centrifuged twice at 10,000g at 4°C for 15min. The supernatants were removed and stored at 4°C prior to the assay of MCP-1, IL-6, and TNF- α via ELISA the following day (R&D Systems, Minneapolis, MN). The assay was performed according to the manufacturer's instructions and all samples were run in duplicate. Total soluble protein was determined using supernatant of homogenized samples via bicinchoninic acid (BCA)

protein assay (Pierce, Rockford, IL.). All cytokine levels are expressed as a pg per 100 µg of total protein.

2.8 mRNA expression

Thoracic mammary gland and mammary tumor tissue from all sites was homogenized under liquid nitrogen and RNA was isolated using TRIzol reagent (Life Technologies, GIBCO-BRL, Carlsbad, CA). RNA was reverse transcribed into cDNA and quantitative RT-PCR was carried out as per the manufacturer's instructions (Applied Biosystems, Foster City, CA) using TaqMan Gene Expression Assays as previously described [26]. Conditions utilized for RT-PCR were as follows: 2 min at 50°C; 10min at 95°C; and 40 repetitions of 15 seconds at 95°C and 1 min at 60°C. Genes measured included MCP-1 (Mm00441242_m1), F4/80 (Mm00802529_m1), IL-10 (Mm00439614_m1), CD206 (Mm00485148_m1), IL-12/p35 (Mm00434165_m1), IL-6 (Mm00446190_m1), TNF-α (Mm00443258_m1) and 18S (Mn03928990_g1) as the reference gene. Quantification of mRNA expression of all target genes (MCP-1, F4/80, IL-10, CD206, IL-12/p35, IL-6, and TNF-α) was calculated using the 2^{-C_T} method, which employs a single calibrator sample to compare every unknown sample's gene expression against. Briefly, $C_T [C_T (FAM) - C_T (VIC)]$ was calculated for each sample and the average C_T of the control mice was used as the calibrator sample. $C_T [C_T (calibrator) - C_T (sample)]$ was then determined for each sample and the relative quantification was calculated as 2^{-CT} . Average CT and delta CT values are presented in Table 2 (mammary gland) and Table 3 (tumor tissue).

2.9 Statistical Analysis

All data were analyzed using commercial statistical software (SigmaStat, SPSS, Chicago, IL). Weekly tumor measurements, body weight, and food and water intake were analyzed using a repeated measures two-way ANOVA (time x group) with Student-Newman-Keuls post hoc testing. Sacrifice data including body weight and spleen weight, as well as mammary gland mRNA expression and protein expression data was analyzed using a two-way ANOVA (strain x treatment) with Student-Newman-Keuls post-hoc testing. When only the effect of bindarit treatment was compared within C3(1)/SV40Tag mice (e.g. in analysis of tumor tissue), one-tailed independent student's t-tests was performed. Analysis of relationships between several outcome measures and tumor volume and number was completed using Pearson product moment correlations. For all measures statistical significance was set at an alpha value of $P < 0.05$. All data are presented as mean $\pm$ SEM.

3. RESULTS

3.1 Treatment and Descriptive Characteristics

3.1.1 Food intake and bindarit dose—Food consumption was similar across all treatment groups. Accordingly, there were no differences in the dose of bindarit received by the FVB and C3(1)/SV40Tag mice. Based on average weekly food consumption and weekly body weight measurements, FVB-Bin mice consumed 714.0 ± 18.6 mg/kg BW per day of bindarit and C3-Bin mice ingested 703.4 ± 22.7 mg/kg BW per day.

3.1.2 Body Weight—Body weight was measured weekly throughout the treatment period (4–21 weeks of age), prior to sacrifice, and after the removal of all tumors. A significant main effect of bindarit treatment was detected between the groups ($P<0.05$) but there was no effect of genotype and no interaction (Figure 1A). Body weight measured at sacrifice prior to the removal of mammary tumors showed no differences between the four groups. However, following the removal of the tumor weight, C3-Bin mice had elevated body weight compared to the C3-Con group ($P<0.05$) (Figure 1B).

3.1.3 Spleen weight—Spleen weight was assessed at sacrifice as it has been previously correlated with tumor burden in this mouse model [21]. A significant main effect of strain (FVB versus C3(1)/SV40Tag) existed for absolute spleen weight (FVB-Con: 97.5 ± 2.6 g; FVB-Bin: 95.9 ± 3.6 g; C3-Con: 146.7 ± 10.1 g; C3-Bin: 143.6 ± 13.9 g), as well as spleen weight expressed as a percentage of body weight (FVB-Con: $0.42 \pm 0.01\%$; FVB-Bin: $0.40 \pm 0.01\%$; C3-Con: $0.66 \pm 0.05\%$; C3-Bin: $0.55 \pm 0.05\%$) ($P<0.05$). No differences were detected between C3(1)/SV40Tag groups for absolute spleen weight, but when expressed relative to body weight, spleen weight was significantly lower in the C3-Bin mice ($P<0.05$). In agreement with our previous findings, spleen weight was significantly correlated with tumor volume and tumor number at sacrifice in the C3(1)/SV40Tag mice ($p<0.01$) (data not shown) [21].

3.2 Bindarit reduces tumor multiplicity, but not size

Bindarit treatment significantly reduced tumor number from 19 weeks of age through sacrifice at 21 weeks of age ($P<0.05$) (Figure 2A). At sacrifice C3-Con mice had an average of 13.9 ± 1.0 tumors while C3-Bin mice averaged 10.7 ± 1.5 tumors, resulting in a 23% decrease in overall tumor number (Figure 2B). However, time (from birth) to the development of the first palpable tumor was not significantly reduced by bindarit (C3-Con: 113.5 ± 3.6 days; C3-Bin: 118.6 ± 2.6 days) (Table 1). Similarly, tumor volume was not different at any point throughout the treatment period or at sacrifice (Figure 3A and 3B); tumor volume at sacrifice in the C3-Con mice was 1165.0 ± 257.1 mm³ and was 1250.8 ± 335.9 mm³ in the C3-Bin mice. Further, no differences in average tumor weight were observed at sacrifice (C3-Con: 1.18 ± 0.28 g; C3-Bin: 1.27 ± 0.34 g) (Table 1).

Thoracic mammary glands were removed and processed for H&E staining to allow for investigation of histopathology (Figure 4). Glands from both C3-Con and C3-Bin mice provided evidence of the presence of intraepithelial neoplasia, ductal carcinoma in situ and very apparent invasive carcinoma formation with no differences between the groups.

3.4 Inflammatory mediator expression in mammary tumor and mammary gland tissue was reduced by bindarit

The effects of bindarit were most pronounced within the tumor tissue as it decreased tumor mRNA expression (28%) and protein concentration (15%) of its target gene, MCP-1 (Figure 5A and 5B) ($P<0.05$). Similarly, tumor protein levels of both TNF- α (47%) and IL-6 (30%) were reduced by bindarit ($P<0.05$) (Figure 5B). Changes in the mRNA expression of these inflammatory cytokines in the tumor tissue was not as marked though, as TNF- α expression

was only reduced 17% and IL-6 by 12% following bindarit treatment (Figure 5A), neither of which reached statistical significance.

Within the thoracic mammary glands evidence of a pro-tumorigenic environment was detected as there was a significant main effect of strain on the mRNA expression of MCP-1, TNF- α , and IL-6; levels were significantly elevated in C3(1)/SV40Tag mice compared to cancer-free FVB/N wildtype mice (Figure 6A). Within the C3(1)/SV40Tag mice, bindarit treatment tended to reduce the expression of each of these markers, though a statistically significant effect was only observed for IL-6 ($P<0.05$).

3.5 Bindarit has nominal effects on the mRNA expression of macrophage markers in the tumor and mammary gland tissue

Tumor associated macrophages express pro-tumor properties including a tendency towards a M2 phenotype characterized by the expression of the mannose receptor, CD206, and IL-10^{high}IL-12^{low}. Within the tumor tissue, the general macrophage marker F4/80 was reduced 15% by bindarit treatment and mRNA expression of CD206 and IL-10 were decreased 23% ($p=0.09$), and 36% ($p=0.13$), respectively (Figure 8A). IL-12, which is also targeted specifically by bindarit, was reduced by more than 60% in the mammary tumor tissue ($P<0.05$) (Figure 7A).

The microenvironment surrounding the tumor is important to its ability to sustain growth. Similar to the inflammatory mediators described above, a significant main effect of strain was detected as several of the macrophage markers (F4/80, IL-10, and IL-12) were elevated in the dysplastic mammary gland tissue of C3(1)/SV40Tag mice compared with both FVB treatment groups (Figure 7B). The mRNA expression of the M2 marker CD206, however, was not different between the four groups. There were also no significant changes in these macrophages markers following bindarit treatment in either of the strains of mice.

3.6 Bindarit has negligible effects on plasma MCP-1 and IL-6

Bindarit was administered through dietary incorporation beginning at 4 weeks of age and was continued until sacrifice at 21 weeks of age at which time plasma MCP-1 and IL-6 were assessed. For both MCP-1 and IL-6 there was a significant main effect for strain as C3(1)/SV40Tag mice had significantly higher plasma levels compared to FVB/N mice ($P<0.05$) (Figure 8A and 9A). However, bindarit treatment did not significantly reduce plasma MCP-1 or IL-6 levels in either strain.

Correlations were performed to assess a potential relationship between both MCP-1 and IL-6, and tumor characteristics. For this analysis the C3(1)-Con and C3(1)-Bin mice were analyzed as one C3(1) group. Tumor volume, weight and number measured at sacrifice were all significantly positively correlated with plasma MCP-1 (Figure 8B, 8C, 8D). The strongest linear relationships existed for tumor volume ($R=0.889$, $P<0.001$), followed by tumor weight ($R=0.885$, $P<0.001$) and tumor number ($R=0.437$, $P=0.02$). Plasma IL-6 levels were also significantly correlated with tumor volume ($R=0.6159$, $P=0.0004$) and tumor weight ($R=0.6305$, $P=0.0002$), but not with tumor number ($R=0.0957$, $P=0.6213$) (Figure 9B, 9C, 9D).

4. DISCUSSION

Observational and experimental evidence supports a role for MCP-1 in the enhancement of breast cancer that is mediated, at least in part, through recruitment and activation of tumor associated macrophages [27]. However, the importance of MCP-1 in the development and progression of breast cancer has not yet been established in the C3(1)/SV40Tag transgenic mouse model. Therefore, using the novel *indazolic* derivative bindarit to target MCP-1, we investigated the importance of this chemokine on tumor establishment and growth in the triple-negative C3(1)/SV40Tag mouse model of breast cancer. Additionally, we examined the effects of bindarit on macrophage markers and inflammatory mediators that are known to be influenced by MCP-1. Results show that C3(1)/SV40Tag mice treated with bindarit experienced a small, but significant, decrease in tumor number but no attenuation of tumor volume. Neither plasma MCP-1 nor IL-6 was not reduced by bindarit treatment; however, evidence of an effect of bindarit was detected within the tumor microenvironment as gene expression and protein concentration of MCP-1 was reduced. Additionally, tumor tissue protein concentration and/or gene expression of several macrophage and inflammatory mediators including IL-6, TNF- α , IL-12 and CD206 were reduced by bindarit. These data support a benefit of bindarit on tumor number in the C3(1)/SV40Tag mouse model of breast cancer that is associated with a reduction in select macrophages markers and inflammatory mediators in the tumor microenvironment.

Normal, disease-free breast epithelial cells lack significant expression of MCP-1 (unless stimulated), while expression is greatly elevated in both neoplastic and stromal cells within the breast tumor microenvironment [7, 9, 14, 15, 28–31]. The expression of MCP-1 is an acquired feature gained during tumor development implying that it is advantageous to tumor establishment. In primary breast tumors, MCP-1 has significant prognostic value for relapse free survival, is significantly correlated with high tumor grade, lymph node metastasis, and is associated with low levels of differentiation and poor prognosis [7, 10, 12, 13, 32]. In the present investigation, we show for the first time that bindarit, an MCP-1 inhibitor, can lead to a significant reduction in mammary tumor multiplicity in the C3(1)/SV40Tag transgenic mouse model of breast cancer. However, despite reducing tumor number, bindarit did not delay the formation of the initial palpable tumor nor slow tumor growth as tumor volume and latency were similar between the C3(1)/SV40Tag groups. Spleen weight was also measured as it has been correlated with tumorigenesis in this mouse model. Bindarit treatment decreased spleen weight in C3(1)/SV40Tag mice when expressed relative to body weight. These results are supported, at least in part, by previous investigations also utilizing bindarit in the treatment of carcinomas [17, 18]. For example, Zollo et al. reported a 50% reduction in local tumor growth following bindarit administration in a 4T1-Luc breast cancer xenograft mouse model [18].

Since the primary target of bindarit is MCP-1, we next examined levels of this chemokine in plasma, mammary tumor tissue and surrounding neoplastic mammary gland tissue. In general, our findings indicate a reduction in MCP-1 in bindarit treated mice. This is consistent with Zollo et al.'s investigation as they also detected a decrease in tumor MCP-1 protein levels [18]. It has also been reported that treatment with an MCP-1 antibody significantly decreased tumor number and size, increased survival and decreased metastatic

lung lesions in a SCID mouse injected with MDA-MB-231 breast cancer cells [33]. Conversely, we did not observe a decrease in circulating levels of MCP-1 as has been previously reported following bindarit treatment in both a rat model of severe acute pancreatitis, and rat and mouse models of hyperlipidaemic vascular injury [34, 35]. Since the disease models and treatment doses employed were different, it is difficult to directly compare the findings. However, it is possible that the long term treatment protocol used in our investigation allowed mice to develop a tolerance to bindarit, or initiate a compensatory response to its suppressive effects. Measurement of MCP-1 at earlier progressive time points would be advantageous in order to determine if levels were initially decreased before normalizing by the sacrifice time point. This possibility is somewhat unlikely though since tissue levels of MCP-1 were significantly attenuated in the C3(1)/SV40Tag mice. Therefore, maintenance of plasma MCP-1 levels in spite of bindarit treatment, may have contributed to the lack of differences in tumor growth between the C3(1)/SV40Tag treatment groups. In support of this hypothesis, highly significant correlations were observed between tumor volume and plasma MCP-1 at sacrifice in bindarit treated C3(1)/SV40Tag mice.

Given that MCP-1 has been suggested to be the most important chemokine for macrophage recruitment to the tumor microenvironment [8] and our findings of a reduction in MCP-1 in the tumor tissue, we examined the effects of bindarit on selected macrophage markers. Overall, bindarit treatment produced moderate changes in the expression of several macrophage markers. Specifically, we found that F4/80 expression was slightly reduced and that the expression of CD206 and IL-10, both markers of M2 (pro-tumor) macrophages, were also marginally decreased in bindarit treated mice. Additionally however, bindarit significantly reduced the expression of the M1 (anti-tumor) macrophage marker, IL-12/p35. This was not entirely surprising given that bindarit also targets IL-12/p40 and IL-12/p35, which when reduced, can enhance immunosuppression and differentiation of M2 macrophages [36]. Accordingly, previous work has shown that an IL-12/pulse IL-2 treatment regime initiated after tumor establishment (~20wks of age) in C3(1)/SV40Tag mice reduced tumor development and growth [37]. Therefore, inhibition of both IL-12 and MCP-1 by bindarit may provide a potential paradoxical effect in cancer where the balance of M1 and M2 macrophages can greatly influence tumorigenesis. This may, at least in part, explain our findings of a lack of an effect of bindarit treatment on tumor volume. The current findings though should be substantiated using fluorescence activated cell sorting (FACS) methodology to firmly establish the role of bindarit on macrophage behavior in the C3(1)/SV40Tag mouse model of breast cancer.

It has been proposed that the mutual interaction of macrophages with cancer cells enhances production of inflammatory cytokines which transform the tumor microenvironment into one which favors the growth, survival and motility of neoplastic cells [10, 38, 39]. The inflammatory cytokines TNF- α and IL-6 have been associated with poor outcome and enhanced tumor growth in breast cancer in addition to being related to tumor macrophage expression of MCP-1 [7, 40]. Given the observed reductions in MCP-1 and macrophage markers with bindarit, we investigated the expression and concentration of TNF- α and IL-6 in the tumor microenvironment and their expression in the surrounding dysplastic mammary tissue. Our findings indicate that a reduction in MCP-1 by bindarit corresponds with decreases in protein concentration of TNF- α and IL-6 in the tumor tissue and mRNA

expression of IL-6 in the mammary gland. A similar relationship has been observed in primary invasive ductal carcinoma breast tissue samples where the expression of MCP-1 was positively correlated with the expression of both IL-6 and TNF- α [7]. Additionally, a phase II clinical trial in women with metastatic breast cancer has shown that treatment with an MCP-1 inhibitor reduced plasma levels of both IL-6 and MCP-1 [41]. However, plasma levels of IL-6 were not reduced following bindarit treatment in the current investigation, which is not surprising given that circulating MCP-1 was not affected and that the effects of bindarit are known to be independent of IL-6 [36, 42].

In contrast to the current findings of a reduction in mammary tumor number with modest decreases in macrophage markers and inflammatory cytokines, bindarit treatment has previously been reported to cause a substantial inhibition of tumor growth, angiogenesis and tumor macrophage infiltration in other mouse models of breast cancer and melanoma [17, 18]. Numerous methodological differences likely contributed to the smaller magnitude of change reported in the current study. Firstly, we used a transgenic mouse model of breast cancer and employed a prevention plus interventional study design in which treatment was initiated prior to and continued through tumor development as opposed to Zollo et al. (2012) who administered interventional bindarit treatment for the duration of 2 weeks in a xenograft mouse model already displaying palpable tumors [17, 18]. Secondly, although the bindarit treatment was well tolerated with similar food consumption and little or no change in body weight across groups, the dose (~700mg/kg BW/d vs. 100–200mg/kg BW/d), as well as the route of administration (dietary incorporation vs. oral gavage or IP injection) differed between our study and those of others [17, 18]. Due to the long-term nature of our study we felt it would have been unduly stressful to the animals to deliver bindarit twice daily for 17 weeks via gavage or IP injection. The current dietary treatment regime was based on previous work citing this dietary dose to cause significant reductions in MCP-1 in shorter term studies. This investigation is the first to give bindarit over a prolonged period of time and raises issues of efficacy which may need to be addressed in future studies. In the current investigation, bindarit treatment did not induce inhibition of plasma MCP-1, which suggests that the overall effectiveness of the treatment dose, timing and administration method may not have been optimal at least in this mouse model. Further experimentation will be required to determine if the lack of reductions in tumor growth following bindarit treatment were related to methodological differences.

In summary, the results of the current investigation provide the first evidence of a benefit of the MCP-1 inhibitor, bindarit, on tumor number in a transgenic mouse model of breast cancer, however this was independent of a reduction in tumor volume. Plasma MCP-1 was not reduced by bindarit treatment but evidence of an effect of bindarit was detected within the mammary gland and tumor tissue as gene and protein expression of MCP-1 was decreased. Further, the expression of select macrophage markers as well as inflammatory mediators (e.g. IL-6, TNF- α) were reduced by bindarit primarily in the tumor tissue. Overall, we report potentially important beneficial effects of bindarit in the C3(1)/SV40Tag mouse model. The smaller magnitude of changes observed in contrast to previous investigations utilizing bindarit is likely due to the different dosage, administration method and/or cancer model employed. Future investigations of the efficacy of long-term oral administration of bindarit treatment at different stages of tumor development and growth are

necessary in order to establish the clinical applicability of this drug in the treatment of breast cancer.

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ABBREVIATIONS

MCP-1	Monocyte chemoattractant protein-1
TAMs	Tumor associated macrophages
MIN	Mammary intraepithelial neoplasia
DCIS	Ductal carcinoma in situ

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Highlights

1. Bindarit significantly decreased tumor number, but not tumor volume in C3(1)/SV40Tag mice.
2. Bindarit reduced the concentration and expression of MCP-1 within the tumor microenvironment.
3. Several macrophage and inflammatory related factors were also reduced by bindarit.

Figure 1. Bindarit increased body weight of C3(1)/SV40Tag mice independent of tumor weight Body weight was measured weekly (A) and at sacrifice following the removal of all mammary tumors (B). Values are means $\pm$ SEM. Figure 1A: a) denotes significant differences between C3-Con and FVB-Bin; b) significant differences between C3-Con and C3-Bin; c) significant differences between C3-Con and FVB-Con; d) significant differences between FVB-Con and FVB-Bin; e) denotes significant differences between FVB-Con and C3-Bin; ($P < 0.05$). Figure 1B: *significantly different from C3-Con, $P < 0.05$.

Figure 2. Bindarit reduced tumor number in C3(1)/SV40Tag mice

Mice were palpated twice a week from 10–21 weeks of age and tumors were counted and measured. (A) Tumor number was significantly reduced from 18.5–21 weeks of age, * $p < 0.05$. (B) At sacrifice all visible tumors were counted in the 10 mammary glands. Values are means $\pm$ SEM.

Figure 3. Bindarit did not reduce tumor volume in C3(1)/SV40Tag mice

Mice were palpated twice a week from 10–21 weeks of age and tumors were measured using calipers for calculation of tumor volume. Tumor volume over time (A) and at sacrifice (B) was not different between the treatment groups. Values are means $\pm$ SEM.

Figure 4. Bindarit did not alter composition of thoracic mammary gland tissue in FVB/N and C3(1)/SV40Tag mice

Thoracic mammary glands were fixed in formalin, embedded and then sectioned for H&E staining. Images were captured using the DAKO Chromavision Systems ACIS 3 system. Glands from FVB-Con (A), FVB-Bin (B), C3-Con (C), and C3-Bin (D) mice are presented. Large areas of invasive carcinoma as well as advanced lesion formation are present in C3-Con and C3-Bin mice. Arrows indicate a few of the large invasive carcinoma formations within the mammary gland.

Figure 5. Tumor MCP-1, TNF- α , and IL-6 expression are reduced by bindarit in C3(1)/SV40Tag mice

Mammary tumor tissue was collected from all mice and processed for either gene expression (A) analysis via RT-PCR or protein expression (B) via ELISAs. Values are means $\pm$ SEM.

*significantly different from C3-Con, ($P < 0.05$); #trend for differences from C3-Con, ($P < 0.1$).

Figure 6. Expression of inflammatory cytokines are elevated in mammary glands of C3(1)/SV40Tag mice

Thoracic mammary gland tissue was collected from all mice and processed for mRNA expression via RT-PCR. Values are means $\pm$ SEM. *significantly different from FVB-Con, ^ significantly different from FVB-Bin, &significantly different from C3-Con; ($P < 0.05$).

Figure 7. Bindarit reduces macrophage related expression of M1 and M2 markers in mammary tumor tissue

Tumor (A) and thoracic mammary gland (B) tissue was collected from all mice and RT-PCR was performed to access mRNA expression of F4/80, CD206, IL-10, and IL-12/p35. Values are means $\pm$ SEM. (A) ^{*}significantly different from C3-Con, ($P < 0.05$); [#]trend for differences from C3-Con, ($P < 0.1$). (B) ^{*}significantly different from FVB-Con, [^]significantly different from FVB-Bin, ($P < 0.05$).

Figure 8. Plasma MCP-1 was not reduced in bindarit treated mice at sacrifice

MCP-1 was measured in the plasma of FVB and C3(1)/SV40Tag mice at 21 weeks of age (sacrifice) (A). Correlation analysis revealed significant associations between MCP-1 and tumor volume (B), weight (C), and number (D) for C3-Con and C3-Bin mice. Values are mean $\pm$ SEM. *significantly different from FVB-Con; $P<0.05$.

Figure 9. Plasma IL-6 was not affected by bindarit treatment

IL-6 was measured in the plasma of FVB and C3(1)/SV40Tag mice at 21 weeks of age (sacrifice) (A). Correlation analysis revealed a significant association between IL-6 and tumor volume (B), and IL-6 and tumor weight (C), but not between IL-6 and tumor number (D). Values are mean $\pm$ SEM. [^] significantly different from FVB-Bin; $P<0.05$.

Table 1
The effects of bindarit on tumor characteristics in C3(1)/SV40Tagmice

Time (from birth) to the development of the first palpable tumor was calculated (days) following twice weekly measurements initiated at 10 weeks of age. At sacrifice all tumors were removed, counted and measured. Tumor volume was calculated using the formula $0.52 \times (\text{largest diameter}) \times (\text{smallest diameter})^2$ and is expressed as mm^3 . Values are means $\pm$ SEM.

	C3-Con	C3-Bin
Latency (days)	113.5 $\pm$ 3.6	118.6 $\pm$ 2.7
Multiplicity (#)	13.9 $\pm$ 1.0	10.7 $\pm$ 1.5
Volume (mm^3)	1165.0 $\pm$ 257.1	1250.8 $\pm$ 335.9
Weight (g)	1.18 $\pm$ 0.3	1.27 $\pm$ 0.34

Table 2
The CT and Delta CT values in the mammary glands of FVB and C3(1)/SV40Tag mice

Taq man gene expression assays were used for quantification of gene expression of target genes. Table 2A: Target gene average CT values. Table 2B: Average CT values of 18S (internal control) for the corresponding target gene listed in the same order as above in Table 2A. Table 2C: The delta (target-internal control) CT values for all genes measured in mammary glands of FVB and C3(1)/SV40Tag mice.

A.				
Target gene	FVB-Con	FVB-Bin	C3-Con	C3-Bin
MCP-1	31.3	32.3	28.6	29.1
F4/80	26.5	26.8	24.7	24.9
CD206	24.5	24.9	24.0	23.9
IL-10	33.4	34.4	32.0	32.3
IL-12	34.7	35.1	33.4	33.7
TNF- α	31.6	32.4	29.3	29.9
IL-6	33.9	34.1	31.4	32.1

B.			
FVB-Con	FVB-Bin	C3-Con	C3-Bin
10.7	10.9	10.2	10.2
10.9	10.9	10.4	10.3
10.9	11.0	10.7	10.5
11.2	11.2	10.7	10.8
11.2	11.3	10.9	10.8
10.3	9.9	9.3	9.6
8.3	8.3	7.9	7.9

C.				
Target gene	FVB-Con	FVB-Bin	C3-Con	C3-Bin
MCP-1	20.6	21.4	18.4	18.9
F4/80	15.6	15.9	14.3	14.7
CD206	13.5	13.9	13.3	13.3
IL-10	22.2	23.1	21.3	21.5
IL-12	23.6	23.7	22.5	22.9
TNF- α	21.2	22.4	19.9	20.3
IL-6	25.5	25.8	23.5	24.2

Table 3

The CT and Delta CT values in tumor tissue of C3(1)/SV40Tag mice. Taq man gene expression assays were used to quantify the expression of several target genes within the tumor tissue. Table 3A: Target gene average CT values. Table 3B: Average CT values of 18S (internal control) for the corresponding target gene listed in the same order as above in Table 3A. Table 3C: The delta (target-internal control) CT values for all genes measured in the tumor tissue of C3(1)/SV40Tag mice.

A.			B.	
Target Gene	C3-Con	C3-Bin	C3-Con	C3-Bin
MCP-1	26.8	26.7	8.3	7.8
F4/80	23.9	23.9	8.5	8.1
CD206	25.4	25.3	8.6	8.2
IL-10	31.6	31.6	8.7	8.4
IL-12	30.0	30.7	8.8	8.4
TNF- α	27.3	27.4	7.7	7.4
IL-6	31.4	30.9	6.3	6.0

C.		
Target Gene	C3-Con	C3-Bin
MCP-1	18.4	18.8
F4/80	15.4	15.8
CD206	22.8	23.3
IL-10	21.2	22.3
IL-12	16.7	17.1
TNF- α	19.6	19.9
IL-6	24.8	25.0