Published in final edited form as:

Cancer Immunol Immunother. 2018 January ; 67(1): 13–23. doi:10.1007/s00262-017-2057-0.

The STAT3 inhibitor pyrimethamine displays anti-cancer and immune stimulatory effects in murine models of breast cancer

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Abstract

The transcription factor signal activator and transducer or transcription (STAT)3, which regulates genes controlling proliferation, survival, and invasion, is activated inappropriately in many human cancers, including breast cancer. Activation of STAT3 can lead to both malignant cellular behavior, as well as to suppression of immune cell function in the tumor microenvironment. Through a chemical-biology screen, pyrimethamine (PYR), an FDA approved anti-microbial drug, was identified as an inhibitor of STAT3 function at concentrations known to be achieved safely in humans. We report that PYR shows therapeutic activity in two independent mouse models of breast cancer, with both direct tumor inhibitory and immune stimulatory effects. PYR inhibited STAT3 activity in TUBO and TM40D-MB metastatic breast cancer cells *in vitro* and inhibited tumor cell proliferation and invasion into Matrigel basement membrane matrix. In tumor-transplanted mice, PYR had both direct and indirect tumor inhibitory effects. Tumor bearing mice treated with PYR showed reduced STAT3 activation in tumor cells, attenuated tumor growth, and reduced tumor-associated inflammation. In addition, expression of Lamp1 by tumor infiltrating CD8⁺ T-cells was elevated, indicating enhanced release of cytotoxic granules. These findings suggest that PYR may have beneficial effects in the treatment of breast cancer.

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Conflict of Interest: The authors declare that they have no conflict of interest.

Keywords

Breast cancer; Metastasis; Immunity; Pyrimethamine; STAT3

Introduction

Signal transducer and activator of transcription (STAT)3 is a member of a family of seven closely related transcription factors. STAT3 is present in the cytoplasm of cells under basal conditions. In response to cytokines or other extracellular stimuli, STAT3 becomes phosphorylated by Jaks or other kinases on a carboxy-terminus tyrosine residues. It then translocates to the nucleus as a dimer, where it can bind to nine base pair regions in the promoters of target genes to regulate transcription [1]. In addition to regulating genes controlling proliferation, survival, and pluripotency, STAT3 activity can contribute to inflammation and immune suppression [2]. In all subtypes of breast cancer, STAT3 can become constitutively activated. In particular, it is commonly up-regulated in the tumor initiating cell population, where it regulates cancer initiation, maintenance, and relapse [3,4].

Given the central role played by STAT3 in the pathogenesis of breast cancer, and other human cancers, efforts have been made to identify clinically accessible inhibitors of this protein. From a screen of a chemical library biased to drugs already known to be safe in humans, pyrimethamine (2,4-diamino-5-p-chlorophenyl-6-ethyl-pyrimidine) (PYR), an anti-microbial that is effective in the prevention and treatment of malaria and toxoplasmosis, was found to inhibit STAT3 transcriptional function at concentrations known to be safely achieved in patients [5,6].

While previous studies have focused on the direct impact of PYR on tumor cells in cell culture systems [7–10], there is little information on the *in vivo* action and immunological impact of PYR in cancer models. STAT3 is activated downstream of cytokine signaling in hematopoietic cells and plays a dual role in tumor inflammation and immunity by stimulating pro-oncogenic inflammatory pathways, including nuclear factor- κ B (NF- κ B) and interleukin-6 (IL-6)–GP130–Janus kinase (JAK) pathways, as well as by antagonizing STAT1- and NF- κ B-regulated T_H1 anti-tumor immune responses [11]. Therefore, the inhibition of STAT3 activity by PYR may have both tumor cell autonomous as well as immune modulatory effects.

Breast cancer is the most frequently diagnosed cancer and the leading cause of cancer-related death among women worldwide [12]. The *HER2* gene, which encodes a tyrosine kinase receptor, is a strong mediator of cellular growth and proliferation in normal as well as malignant epithelial cells [13]. *HER2/neu* overexpression occurs in 20% to 30% of breast cancer cases and is associated with inflammatory tumors and poor prognosis [13]. MMTV-promoter-*NeuT* transgenic mice faithfully reproduce the tumor and immune pathology of *HER2+* breast cancer and breast cancer metastasis [14–16]. The TM40D-MB is a highly metastatic and well-characterized breast tumor cell line that does not express *HER2* [17].

We report protective effects of PYR through inactivation of STAT3 in tumor cells and enhanced tumor immunity in mouse models of breast cancer. In these studies we used the

BALBc-NeuT transgenic mice as well as mice transplanted with TUBO cells that were originally derived from a lobular carcinoma that arose spontaneously in a BALBc-neuT mouse [18]. The efficacy of PYR as a therapeutic drug was validated in mice transplanted with TM40D-MB, an unrelated breast cancer cell line with high metastatic potential. We show that the protective effects of PYR are mediated in part through suppressing cancer-associated inflammation and reducing the immunosuppressive tumor microenvironment, thereby leading to the activation of anti-tumor immunity and effector functions of cytotoxic T-cells.

Materials and Methods

Cell culture

TUBO cells [19] were cultured in DMEM (ThermoFisher) supplemented with 5% fetal bovine serum. TM40D-MB cells were grown in DMEM/F12 medium with 2% ABS, epidermal growth factor, and insulin, as described previously [20]

BALB/c-TUBO transplant Breast cancer Mouse model (BTBM)

TUBO cells at 40–50% confluence were trypsinized, washed 2X with phosphate buffered saline (PBS), and triturated into a single cell suspension in PBS. TUBO cells (1×10^6) were injected in the left mammary fat pad of 6-week-old virgin BALB/c females and were monitored for 1 week for visible tumor growth. One week following implantation, mice were treated by gavage with either PYR (60mg/kg/day in NMP (1-Methyl-2-pyrrolidinone):Poly (ethylene glycol)-300 in 1:9 ratio) or vehicle (NMP (1-Methyl-2-pyrrolidinone) and Poly (ethylene glycol)-300 in 1:9 ratio) for 41 days.

BALB-neuT transgenic mice

Inbred BALB/c mice overexpressing the transforming rat Her-2/*neu* oncogene (neuT⁺/neuT⁻) driven by the mouse mammary tumor virus promoter (BALB-neuT), and transgene negative (neuT⁻/neuT⁻) (BALB/c) were produced and screened for the presence of the transgene as previously described [18]. Mice were treated with either PYR (60mg/kg/day) or carrier for 48 days.

For all murine experiments, mice were maintained under specific pathogen-free conditions at the Northwestern University Animal Care Facility, and the Animal Care Usage Committee of Northwestern University approved all experiments. Tumors were measured with calipers in two perpendicular diameters.

Western Blotting

Whole-cell extracts were prepared in RIPA buffer (10mM Tris-HCl [pH 7.5], 500mM NaCl, 0.1 % SDS, 1% NP-40, 1% sodium deoxycholate, 2mM EDTA, and 1% protease, phosphatase I and II inhibitor cocktail [Sigma]). Cellular protein (30ug) was resolved by SDS-PAGE, and transferred following standard protocols. Immunoreactive proteins were detected with antibodies to phospho-STAT3 (Tyr705), total STAT3 (Cell Signaling) and GAPDH (Sigma) using appropriate HRP-conjugated secondary antibodies and SuperSignal chemiluminescent reagent (Thermo Scientific).

Immunohistochemistry and Immunofluorescence

4 μ m thick paraffin embedded tissue sections were deparaffinized, and antigen-retrieval was performed using Target retrieval solution (Dako, Carpinteria, CA) in Decloaking chamber. Sections were then blocked with 1%BSA and incubated with anti-pSTAT3-Y705 (Abcam), anti-F4/80 (Abcam) or anti-Ki-67 (Abcam) overnight at 4°C. The sections were then incubated with anti-rabbit-horseradish peroxidase-labeled polymer (Dako) for 1 hour at room temperature, followed by DAB substrate (Dako), counterstaining with hematoxylin, and mounting with PermOUNT mounting medium (for immunohistochemistry). For double-immunofluorescence, after antigen-retrieval and blocking with 1%BSA, staining was performed with anti-Foxp3 (ebiosciences) and anti-ROR γ T (Abcam), or anti-CD8 (Abcam) and anti-Lamp1 (Abcam). Antibodies were applied on tissue sections overnight at 4°C, followed by anti-rat Alexaflor-594 and anti-rabbit Alexaflor-488 for 1 hour in the dark at room temperature followed by DAPI (Invitrogen), and mounted as described [21].

Preparation of conditioned medium

For the production of TUBO conditioned medium (TUBO CM), TUBO cells were grown to 100% confluency in DMEM and treated with either 100 μ M S3I-201 (inhibitor of STAT3 activity, Calbiochem) or vehicle [22], washed three times with PBS, and incubated with fresh DMEM medium for 48 hrs. The conditioned medium (designated either “TUBO CM (+S3I-201)” or “TUBO CM (+DMSO)”) was removed, filtered, and used for cell migration experiments.

Cell proliferation assay

1×10^4 TM40D-MB cells were seeded in triplicate in 100 μ l per well in a 96 well plate with standard medium for 24 hours at 37°C and 5% CO₂. The next day the medium was removed and 100 μ l of either DMEM/F12 with carrier or DMEM/F12 with 10 μ M PYR or DMEM/F12 with 20 μ M PYR was added and incubated for an additional 24, 48 or 72 hours at 37°C and 5% CO₂. 0.5mCi of [³H]-thymidine was added to each well and incubated for 6 hours after the 24, 48 and 72 hours time points. [³H]-thymidine incorporation was quantified using a scintillation counter (LKB RackBeta; Wallac) as described [21].

Cell invasion assay

3×10^4 TM40D-MB cells were treated with either vehicle or 10 μ M PYR or 20 μ M PYR, and 3×10^4 TUBO cells were either treated with either carrier or 100 μ M S3I-201 or 20 μ M PYR. Subsequently, these cells were washed three times with PBS and seeded in triplicate in 500 μ l per well of their respective serum free-standard media in the top wells (inserts) in a 24 well (12) insert plate (BD Biocoat). The bottom well contained the appropriate serum-containing standard media for each cell line. After 48 hours of incubation at 37°C the non-invading cells were removed; after fixing the membrane and staining with Diffquick, invading cells were counted using a bright field microscope as described [21].

Statistical analysis

Statistical analysis between study groups was performed using the Student *t* test, One-way ANOVA or Two-way ANOVA. *P* values less than 0.05 were considered statistically significant.

Results

PYR inhibits phosphorylation of STAT3 in breast cancer cell lines and tumors

In both TUBO and TM40D-MB cells, STAT3 was phosphorylated on Y705, reflecting functional activation (Figure 1a and 1b). Following treatment with PYR, phosphorylation of STAT3 decreased in both cell types, though it is not completely abrogated. This is consistent with the finding that PYR decreases STAT3 transcriptional function through both phosphorylation-dependent and phosphorylation-independent mechanisms. Furthermore, *in vivo* treatment of BTBM mice with PYR via gavage significantly inhibited the STAT3 phosphorylation in both mammary tumors (Figure 1c) and spleen (Figure 1d), as assessed by immunohistochemistry). Taken together, these data show that PYR inhibits STAT3 in breast cancer cell lines, mammary tumors, and immune organs.

Inhibition of pSTAT3 by PYR suppresses breast cancer cell proliferation

Enhanced expression of HER2 protein is associated with more rapid tumor growth [23,24]. Increased tumor cell proliferation has also been directly associated with poor clinical outcome of breast cancer patients [25,26]. HER2 protein tyrosine kinase activity can lead to phosphorylation of STAT3 by both direct and indirect mechanisms. We first assessed the effect of PYR on Her2-overexpressing cell lines *in vitro*. PYR led to a dose-dependent decrease in relative viable cell number, with IC₅₀ values in the low micromolar range (Supplementary Figure 1). Furthermore, at sub-lethal concentrations, PYR decreased expression of a panel of STAT3 target genes in both cell types (Supplementary Figure 2). Micromolar concentrations of PYR are routinely achieved in patients taking this drug for infectious diseases continuously for months without undue toxicity. Therefore, we next studied the *in vivo* impact of STAT3 inhibition by PYR on tumor growth in BTBM mice and BALB-NeuT mice. PYR treatment significantly reduced tumor growth in BTBM mice as well as BALB-NeuT mice in comparison with vehicle-treated mice (Figure 2a and b). Since STAT3 target genes, such as cyclin D1, regulate cell cycle progression, we determined the effect of PYR on proliferation, using Ki-67 staining. We found that tumors from PYR treated BTBM mice display significantly lower proliferation rates (measured by Ki-67 expression) in comparison to the tumors from vehicle-treated BTBM mice (Figure 2c). To complement this finding, we also studied the effect of inhibition of STAT3 on TM40D-MB cell proliferation rates in an *in vitro* thymidine incorporation assay. We observed that 10 μ M PYR significantly lowered the proliferation rates of TM40D-MB cells in comparison to the control group 48 and 72 hours post treatment (Figure 2d). Furthermore, 20 μ M PYR significantly lowered the proliferation rates of TM40D-MB cells at 24, 48 and 72 hour time points in comparison to control as well as 10 μ M PYR treatment group (Figure 2d), indicating dose dependent inhibition of proliferation by PYR.

These findings indicate that PYR-mediated inhibition of STAT3 leads to reduction in the growth and proliferation of Her2/*neu* overexpressing TUBO breast tumor cells and TM40D-MB metastatic breast cancer cells, and the corresponding tumors in mice.

PYR attenuates *in vitro* invasion of TUBO and TM40D-MB cells

In several types of cancer, including breast cancer, the HER2-dependent signaling pathway aberrantly accelerates cell invasion and proliferation of tumor cells and stimulates the epithelial–mesenchymal transition (EMT) necessary for metastases [27–29]. Hence, we studied the effect of STAT3 inhibition on invasiveness of TUBO and TM40D-MB cells. We found that PYR significantly attenuated the invasion of TM40D-MB cells in a dose-dependent manner (Figure 3a and 3b). In addition, we found that PYR significantly inhibited invasion by TUBO cells (Figure 3c and 3d). To determine if other STAT3 inhibitors shared these properties of PYR, we performed a parallel experiment using S3I-201 (100 μ M). This compound produced a similar decreased invasion of TUBO cells (Figure 3c and 3d). We conclude that inhibition of STAT3 function blocks invasion by Her2/*neu* overexpressing TUBO breast tumor cells as well the TM40D-MB metastatic breast cancer cells.

Blocking STAT3 inhibits tumor infiltration by F4/80⁺ macrophages

Over the past few decades, compelling evidence has emerged demonstrating that the immune system participates both in tumor development (via chronic inflammation orchestrated by the innate immune system) and in tumor elimination and control (through the actions of the adaptive immune system). Tumor-associated macrophages (TAMs) consist mainly of a polarized M2 (F4/80⁺/CD206⁺) macrophage population with little cytotoxicity for tumor cells, because of their restricted production of nitric oxide and pro-inflammatory cytokines [30]. TAMs also have poor antigen-presenting capability and effectively suppress T-cell activation. Moreover, TAMs promote tumor cell proliferation and metastasis by secreting a wide range of growth factors, angiogenic factors, and metalloproteinases, and by their involvement in signaling circuits regulating the function of fibroblasts in the tumor stroma [31]. Hence we investigated the population of F4/80⁺ TAMs in BTBM treated with PYR. We found that *in vivo*, STAT3 inhibition significantly attenuated F4/80⁺ TAM infiltration of transplanted TUBO tumors (Figure 4a and 4b). Next we examined migration of spleen-derived mononuclear cells (MNC) in response to conditioned medium from TUBO tumor cells. We found that S3I-201 treatment of TUBO cells significantly inhibited migration of MNC in response to TUBO cell conditioned medium (Figure 4c and 4d). Taken together, these findings indicate that PYR treatment lowers the density of MNC/TAMs in breast cancer tumors, and that inhibition of STAT3 blocks migration of Her2/*neu* overexpressing TUBO breast tumor cells.

Inhibition of STAT3 by PYR mitigates regulatory T-cell/ T-helper-17 immune markers and enhances exocytosis of cytotoxic granules from cytotoxic T-lymphocytes in BALB-NeuT mice

Regulatory T-cells have a dual role in cancer. They are a negative prognostic marker in breast cancer in part due to their ability to suppress anti-tumor immunity [32]. However, they can also protect against cancer by suppressing tumor-associated inflammation [33]. Our earlier studies showed that regulatory T-cells (Tregs) that gain expression of the transcription

factor retinoic acid related orphan receptor gamma T (ROR γ T) lose their ability to suppress cancer associated inflammation [34] and are potently tumor promoting [33]. Hence, our next goal was to study the *in vivo* impact of PYR-mediated STAT3 inhibition on regulatory T-cell / T-helper-17 (Treg/TH17) cells as well as CD8⁺ T-cell mediated cytotoxicity. We found that PYR treatment significantly lowered the frequencies of Foxp3⁺ROR γ T⁻ Tregs (Figure 5a and 5b) as well Foxp3⁺ROR γ T⁺ T-cells (Figure 5a and 5c) in the BALB-NeuT mice. This reflects a decrease in both pro-inflammatory and anti-inflammatory Tregs, which yields an overall effect favoring anti-tumor T-cell cytotoxicity. By contrast, there was no difference in the frequency of CD8⁺ T-cells infiltrating the tumors with PYR treatment (data not shown). However, we found that PYR treatment significantly elevated the numbers of CD8⁺ T-cells that were releasing their cytotoxic granules by exocytosis as measured by Lamp1 staining (Figure 6a and 6b, $P < 0.0001$). Hence, it can be concluded that PYR mediated inhibition of STAT3 restricts Tregs as well as TH17-associated immune responses, and promotes CD8⁺ T-cell mediated cytotoxicity, thus attenuating tumor growth.

Discussion

Constitutive activation of STAT3 occurs in a majority of breast cancers and many other human tumors. That STAT3 directly contributes to oncogenesis has been shown by a number of techniques, including tissue-specific deletion of STAT3, dominant negative STAT3 constructs, and anti-sense and short hairpin RNA [35,36]. Since loss of STAT3 can be tolerated in normal tissue, STAT3 inhibition has the potential to have a high therapeutic index. Using a high-throughput, cell-based assay for specific inhibition of STAT3, PYR was identified as an inhibitor of STAT3 transcriptional function at concentrations known to be routinely achieved in humans without toxicity. HER2⁺ breast cancer and mouse models of mammary tumors driven by HER2 amplification are critically dependent on the HER2-IL-6-STAT3 signaling pathway [37]. Thus, inhibiting STAT3 activity is likely to be an important therapeutic modality for these cancers. STAT3 activation has also been shown to support cancer stem cells in triple negative breast cancer [38–41]. Thus, the therapeutic significance of PYR and other STAT3 inhibitors may expand beyond the HER2 positive group. Testing the effect of PYR on a triple negative breast model could potentially further enhance the clinical importance of the compound.

In the present study we investigated the efficiency of PYR [6], an FDA approved anti-microbial drug, for inhibiting STAT3 activity, tumor associated inflammation and immunity, and tumor growth in mouse models of breast cancer. We focused on HER2⁺ breast cancer in view of the existing evidence linking HER2 to a systemic inflammatory mechanism that regulates tumor growth [37]. IL-6 secretion induced by HER2 overexpression turns on STAT3 and alters gene expression, enforcing an autocrine loop of IL-6/STAT3 expression. To validate our findings and extend these to HER2 negative breast cancer, we used the TM40D-MB metastatic breast cancer model.

Here, we show that PYR inhibits STAT3 activity in Her2/Neu overexpressing TUBO cells, as well as in the tumor and spleen of mice transplanted with TUBO breast cancer cells. STAT3 is activated in response to cytokines and growth factors, through phosphorylation of tyrosine 705 (Y705) by receptor-associated Janus kinases (JAK) and non-receptor tyrosine

kinases of the Src family [42,43]. Dimers of phosphorylated STAT3 translocate to the cell nucleus where they activate transcription of genes mediating cell proliferation and survival, as well as genes that encode immune modulators and growth factors [44]. PYR treatment inhibited proliferation of TM40D-MB metastatic breast cancer cells *in vivo* and invasion of both TM40D-MB and TUBO cells into Matrigel *in vitro*, in a dose dependent manner. Furthermore, we observed reduced numbers of pSTAT3 positive cells in the tumors and spleen of tumor bearing mice that had been transplanted with TUBO cells. These findings demonstrate the ability of PYR to inhibit STAT3 activity in breast cancer cells *in vitro* as well as in breast tumor cells *in vivo*.

The tumor promoting properties of STAT3 have been attributed to regulation of cell proliferation. We showed in mouse models of breast tumor transplant and spontaneous tumor growth that PYR had notable inhibitory effects on tumor growth. This was in part related to reduced tumor cell mitotic activity *in vivo*, and could be reproduced by treatment of either TUBO or TM40D-MB cells in *ex vivo* culture. Further, down regulating STAT3 activity reduced tumor cell proliferation *in vitro* and decreased breast tumor growth *in vivo*. These findings are consistent with earlier reports on the effects of PYR on apoptosis, cell cycle distribution, and cell proliferation in human metastatic melanoma cell lines. In those experiments, PYR used at a clinically relevant concentration induced apoptosis in metastatic melanoma cells by activating cathepsin B and the caspase cascade (i.e., caspase-8 and caspase-9) and subsequent mitochondrial depolarization. Moreover, PYR caused S-phase cell cycle arrest and inhibited cell growth. In addition, results obtained in severe combined immunodeficiency (SCID) mice injected subcutaneously (SC) with human metastatic melanoma cells and treated with PYR, showed an inhibitory effect on tumor growth [9]. PYR treatment was also reported to inhibit telomerase activity [45]. Hence, inhibition of tumor cell growth may be due to multiple mechanisms.

JAK-STAT3 signaling promotes invasion and metastasis by activating several key genes such as WASF3 [46] and MMP-2 [47]. WASF3 [46] is involved in actin cytoskeleton dynamics [48] by recruiting the ARP2/3 complex [49,50], which subsequently upregulates actin polymerization. We found that PYR inhibits invasion of breast cancer in *in vitro* assays, consistent with a role of STAT3 in tumor invasion.

It has become increasingly clear that tumor cells thrive in a chronically inflamed microenvironment and evade immune recognition [51]. Interventions to shift the balance from pro-tumor to anti-tumor immunity offer novel opportunities to control cancer development and progression. Pro-tumor inflammation marked by increased tumor associated macrophages (TAMs) can foster local invasion by supplying matrix-degrading enzymes such as metalloproteinases and cysteine cathepsin proteases [52]. We showed that PYR inhibited TAMs *in vivo* and suppressed migration of monocytes in *ex vivo* assays. Therefore, PYR has anti-inflammatory action and inhibition of cancer-associated inflammation is likely to contribute to the anti-tumor effects of PYR.

Tregs are considered to be a major obstacle to tumor rejection by cytotoxic T-lymphocytes (CTLs) and suppress the action of CTLs by inhibiting granule exocytosis (marked by Lamp1) [53]. We reported earlier that Tregs also have anti-tumor properties related to their

ability to suppress cancer-associated inflammation, in an IL-10 dependent manner. However, we also showed that in progressive cancer, Tregs lose their ability to suppress inflammation while maintaining their T-cell suppressive properties [54,33,55,56]. This change in function was attributed to the upregulation of the canonical TH17 transcription factor ROR γ T in Tregs. STAT3 activity contributes to the upregulation of ROR γ T, expression of IL17, and to the TH17 response, which includes the recruitment of pro-tumor TAMs. In the current study we demonstrated inhibition of both ROR γ T⁻ and ROR γ T⁺ Tregs in tumor bearing mice upon treatment with PYR. The drop in Treg frequency correlates with increased densities of LAMP1⁺ CD8 T-cells. These findings are consistent with the notion that PYR antagonizes Treg function and favors tumor cell lysis by release of cytotoxic granules from CTLs. These findings suggest that by decreasing cancer-associated inflammation and immunosuppression while enhancing effector activity of CTLs, a STAT3 inhibitor would be a particularly attractive and promising therapeutic strategy for treating breast cancer and other malignancies.

In conclusion, we have presented data indicating that PYR has STAT3-inhibitory activity in breast cancer cells and inhibits tumor cell growth *in vitro* and *in vivo*. Furthermore, we have shown that treatment of tumor bearing mice with PYR leads to a reduction in the number of TAMs, Foxp3⁺ Tregs, and Foxp3⁺ROR γ T⁺ T-cells, and an increase in the Lamp1 marker on CD8⁺ T-cells. Thus, PYR inhibits the growth of breast tumors directly by acting on the cancer cells as well as indirectly by shifting a pro-tumor immune response to an anti-tumor immune response. These findings suggest that it may be worthwhile to conduct a clinical trial of PYR, alone or in conjunction with other therapies, for the treatment of advanced breast cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

K. Khazaie was supported by NIH, R01CA160436-05, R01 AA023417-03. F. Gounari was supported by NIH, R01 AI 108682-02.

Abbreviations

BSA	Bovine serum albumin
BTBM	BALBL/c TUBO transplant breast cancer mouse model
CTLs	cytotoxic T-lymphocytes
FDA	United States Food and Drug Administration
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HER2	Epidermal growth factor receptor 2
IL-6	Interleukin 6

JAK	Janus kinase
NMP	1Methyl-2-pyrrolidinone
PBS	Phosphate buffered saline
Poly	Polyethylene glycol
pSTAT	phospho-STAT
PYR	Pyrimethamine
2	4-diamino-5-p-chlorophenyl-6-ethyl-pyrimidine
RORγT	Retinoic acid related orphan receptor gamma T
STAT	Signal activator and transducer of transcription
TAMs	Tumor associated macrophages
TH17	T-helper 17
Tregs	Regulatory T-cells

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Figure 1. PYR decreases the phosphorylation of STAT3 *in vitro* and *in vivo*

(a) TUBO and (b) TM40D-MB breast cancer cells were treated for one hour with vehicle or 10 μ M PYR, after which the indicated proteins were detected by western blot. The ratios of pSTAT3 to STAT3 normalized relative to GAPDH are shown (quantified using ImageJ). (c,d) BTBM mice were treated with vehicle or PYR, and pSTAT3 was detected by immunohistochemistry in tumors (c) or spleen (d); representative immunohistochemistry images (400 $\times$ magnification; scale bar = 20 μ m, arrows indicate nuclear pSTAT3 staining). (e,f) Quantification of pSTAT3 expressing cells in tumor and spleen, respectively (mean $\pm$ standard error; n=7). Tumor sections from a minimum of 3 mice per group were stained for pSTAT3. A minimum of 3 high-power field (400X) images were taken per section (areas of duplication were avoided) and pSTAT3 positive cells per high power field were counted in each group. ** $P < 0.001$ and *** $P < 0.0001$, by Student's t test.

Figure 2. PYR treatment *in vitro* and *in vivo* decreases growth and proliferation of breast cancer cells

(a) BTBM mice with transplanted TUBO mammary tumors (n=14 and (b) BALB/c-NeuT transgenic mice (n=14) with palpable tumors were randomized into arms of treatment with vehicle (Control; red lines) or PYR (blue lines) and tumor size was quantified. (c) Representative images (200 $\times$ magnification, scale bar = 50 μ m) of TUBO tumors on day 41 post transplant, and quantification (brown nuclear staining, mean $\pm$ standard error) of Ki-67 immunohistochemistry of tumor sections. (d) TM40D-MB cells were treated starting from one day post transplant for the indicated times with vehicle or the indicated concentration of PYR, and proliferation was measured by thymidine incorporation; data represent mean $\pm$ standard error. * $P < 0.05$, ** $P < 0.001$ and *** $P < 0.0001$, with two-way ANOVA used in (a) and (b), Student's t test used for (c), and one-way ANOVA used in (d).

Figure 3. STAT3 inhibitors decrease *in vitro* breast cancer cell invasion

(a, b) TM40D-MB cells were treated for one hour with vehicle or the indicated concentration of PYR and membrane invasion was analyzed by microscopy (200× magnification, scale bar = 50 μm) (a) and quantified (mean ± standard error) (b). (c, d) TUBO breast cancer cells were treated for one hour with vehicle or the indicated STAT3 inhibitor, washed and membrane invasion was analyzed by microscopy (200× magnification, scale bar = 50 μm) (c) and quantified (mean ± standard error) (d). ** $P < 0.001$ and *** $P < 0.0001$, by one-way ANOVA.

Figure 4. PYR treatment inhibits migration of F4/80⁺ TAMs *in vivo* and *in vitro*
(a, b) Infiltration of transplanted breast tumors by TAMs *in vivo*. BTBM mice (n=7) were treated with vehicle or PYR for 41 days. Formalin fixed tumor sections were immunostained for infiltrating F4/80 TAMs. Representative images (100× magnification) are shown in (a) and quantification (mean ± standard error) is shown in (b). (c, d) Migration of leukocytes in response to tumor conditioned medium *in vitro*. Splenocyte MNCs were treated with conditioned medium from vehicle- or S3I-201-treated TUBO cells, and migration was determined compared to serum-free DMEM medium as control. Representative images (100× magnification) are shown in (c) and quantification (mean ± standard error) is shown in (d). P values shown were calculated with Student's t test.

Figure 5. PYR treatment lowers the levels of Foxp3⁺ and Foxp3⁺RORγT⁺ cells in mammary tumors

BALB-NeuT mice (n=7) were (a) treated with vehicle or (b) PYR, and Foxp3⁺ (green), RORγT⁺ (red) and Foxp3⁺RORγT⁺ (yellow/merged) cells were visualized by immunofluorescence. White arrow indicates Foxp3⁺RORγT⁺ cells; scale bar = 50 μm. (c) Quantification (mean ± standard error) is shown for the Foxp3⁺RORγT⁻ and Foxp3⁺RORγT⁺ cells in the mammary tumors (n=7). A minimum of 5 high-power field images per mouse (200x) were acquired and Foxp3⁺RORγT⁻ or Foxp3⁺RORγT⁺ cells per high power field were counted. Areas of overlap or duplication were avoided. ** $P < 0.001$ and * $P < 0.05$, by Student's *t* test.

Figure 6. PYR treatment increases densities of tumor infiltrating CD8⁺Lamp1⁺ cells
BALB-NeuT mice (n=7) were treated with (a) vehicle or (b) PYR, and CD8⁺ (red), Lamp1⁺ (green) and CD8⁺Lamp1⁺ (yellow/merged) cells were visualized by immunofluorescence. A minimum of 5 high-power field images per mouse (200×) were acquired and CD8⁺ Lamp1[−] or CD8⁺Lamp1⁺ cells per high power field were counted. Areas of overlap or duplication were avoided. Scale bar = 50 μm. (c) Quantification of CD8⁺Lamp1⁺ cells (mean ± standard error). *** $P < 0.0001$, by Student's t test.