Abstract

Aberrant activation of the latent transcription factor STAT3 and its downstream targets is a common feature of epithelial-derived human cancers, including those of the gastrointestinal tract. Mouse models of gastrointestinal malignancy implicate Stat3 as a key mediator of inflammatory-driven tumorigenesis, in which its cytokine/gp130/Janus kinase (JAK)-dependent activation provides a functional link through which the microenvironment sustains tumor promotion. Although therapeutic targeting of STAT3 is highly desirable, such molecules are not available for immediate clinical assessment. Here, we investigated whether the small-molecule Jak1/2 inhibitor AZD1480 confers therapeutic benefits in two mouse models of inflammation-associated gastrointestinal cancer, which are strictly dependent of excessive Stat3 activation. We confirm genetically that Cre-mediated, tumor cell–specific reduction of Stat3 expression arrests the growth of intestinal-type gastric tumors in gp130F/F mice. We find that systemic administration of AZD1480 readily replicates this effect, which is associated with reduced Stat3 activation and correlates with diminished tumor cell proliferation and increased apoptosis. Likewise, AZD1480 therapy also conferred a cytostatic effect on established tumors in a colitis-associated colon cancer model in wild-type mice. As predicted from our genetic observations in gp130F/F mice, the therapeutic effect of AZD1480 remains fully reversible upon cessation of compound administration. Collectively, our results provide the first evidence that pharmacologic targeting of excessively activated wild-type Jak kinases affords therapeutic suppression of inflammation-associated gastrointestinal cancers progression in vivo.

Introduction

Aberrant activation of the latent transcription factor STAT3 is a common molecular characteristic of many epithelial-derived human cancers (1). STAT3 plays a critical role in controlling transcriptional programs that fuel many processes necessary for tumor promotion (2). Although STAT3 activation is observed in response to various growth factors and cytokines, the interleukin (IL)-6 family of cytokines, defined by their shared use of the GP130 receptor β-chain, arguably play the most important role during tumor promotion (3). Upon ligand engagement of the receptor, the GP130-associated Janus kinases (JAK) JAK1, JAK2, and TYK2 undergo activation before recruiting and phosphorylating cytoplasmic STAT3 to promote its dimerization, nuclear translocation, and transcriptional activity (4).

Because oncogenic mutations in the GP130/JAK/STAT3 pathway occur infrequently in human cancers, aberrant STAT3 signaling predominantly results from excessive autocrine and paracrine signaling by IL-6 family cytokines, making this pathway a prime candidate for therapeutic interference (2). Although STAT3 is a common signaling node for all of the redundantly acting IL-6 family cytokines, small molecules that disrupt STAT3’s protein–protein or protein–DNA interactions with sufficient specificity have not yet been developed for clinical use (5). An alternative, and immediately available approach exists in the inhibition of upstream activators including JAK kinases, for which several small-molecule inhibitors undergo advanced clinical testing for diseases associated with constitutively activating JAK mutations (6). However, because these JAK inhibitors are competitive antagonists of the enzyme’s ATP-binding sites, they also inhibit activity of the corresponding wild-type kinases.

Here, we pursue this notion with the Jak1/2-specific inhibitor AZD1480 (7) in two models of inflammation-associated and Stat3-driven gastrointestinal cancers in mice. We show that therapeutic Jak inhibition confers an...
unexpected cytostatic benefit in situations in which tumor promotion depends on excessive activation of the gp130-associated wild-type forms of these kinases.

Materials and Methods

Mice

All mice were on a mixed C57BL/6 × 129Sv background and maintained under specific pathogen-free conditions. Construction of the transgenic gpa33-CreERT2 strain will be described elsewhere (M. Buchert and M. Ernst; unpublished data), and gp130F/F (8), R26LacZ (9), and Stat3fl/fl (10) mice have been described before. For experiments, littermates were randomly assigned to individual treatment cohorts. Tumor-bearing mice received either AZD1480 (30 mg/kg; AstraZeneca; diluted in 0.5% hydroxypropyl methylcellulose (Dow; #009004-65-3)/0.1% Tween-80 (Fisher; #BP338-500) daily by oral gavage for 6 weeks or diluent alone. Colitis-associated colon cancer (CAC) was induced and monitored by endoscopy as described previously (11–13) with the exception that we administered dextran sodium sulfate (DSS) at a concentration of 2% (w/v) in drinking water. Collection and processing of stomach and intestine was conducted as described previously (12, 13). All animal experimentation was approved by the Ludwig Institute for Cancer Research/Department of Surgery, Royal Melbourne Hospital (Melbourne, VIC, Australia) Animal Ethics Committee.

Immunohistochemistry

Immunohistochemical analysis of gastric and colonic tissue was performed as described previously (12, 13). Apoptosis was detected with the ApopTag Peroxidase Detection Kit (Millipore) according to the manufacturer’s protocol.

qPCR analysis

Tissue was disrupted in TRizol (Invitrogen) using a Qiagen TissueLyser and RNA prepared as described previously (12). cDNA was synthesized by using an Applied Biosystems high-capacity cDNA reverse transcription kit, in which each reaction contained 2 μg of total RNA. We performed quantitative PCR (qPCR) on a Corbett Research Rotogene RG3000, or for the expression of Il6 and Il11 on an Applied Biosystem 7300 Real Time PCR system using TaqMan probes (Applied Biosystems) as described previously (12, 13). Expression was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and we used the 2^−ΔΔCT method to calculate the fold-change. Mann–Whitney U tests were used to determine the statistical differences between ΔCT values and P ≤ 0.05 were considered as significant. Primer sequences are detailed in Supplementary Table S1.

Protein analysis

Protein lysates from whole tissue was prepared as described using radioimmunoprecipitation assay (RIPA) buffer, and 40 μg were separated by PAGE and transferred to nitrocellulose membranes as described previously (12, 13). Membranes were blocked with 5% bovine serum albumin and were incubated with primary antibodies (detailed in Supplementary Table S2) overnight and the appropriate secondary antibodies. Signals were visualized with a Chemidoc XRS+ (Bio-Rad) chemiluminescence detection system.

Statistical analyses

All data are expressed as mean ± SEM. Differences between treatment groups were analyzed by two-sided Student t test.

Results

Genetic limitation of epithelial Stat3 expression inhibits gastric tumor promotion in gp130F/F mice

To test the therapeutic potential of Jak inhibition in an inflammation-associated epithelial cancer model, we used the gp130F/F-mutant mouse as a validated preclinical model of intestinal-type gastric cancer that shares molecular and histologic features with the human disease (12). Although systemic reduction of Stat3 expression in gp130F/F;Stat3−/− mice delays the onset and reduces the growth of tumors (8), it remains unclear whether this is directly attributable to excessive Stat3 activity in the metastatic epithelium of tumors gp130F/F+. We therefore made use of our observation that the intestine-specific glycoprotein gpA33 (14) is expressed in the metastatic epithelium of gp130F/F+, but not in the surrounding non-neoplastic glandular epithelium of the antrum (Supplementary Fig. S1C). Therefore, we generated a novel transgenic mouse strain based on a bacterial artificial chromosome (BAC) that includes all the cis-regulatory elements of the gpa33 gene locus to drive expression of a tamoxifen-dependent CreERT2 DNA recombinase. We then generated corresponding compound gpa33-CreERT2gp130F/F,R26lacZ/+–mutant mice, and following tamoxifen administration, we detected LacZ reporter activity only in the tumor epithelium but not in the adjacent normal glandular epithelium of the antrum (Supplementary Fig. S1C). As expected, we also detected recombination in the intestinal epithelium including the duodenum (data not shown).

We next induced Cre activity in 8-week-old gpa33-CreERT2gp130F/F;Stat3−/− mice with established tumors (Fig. 1A), and as anticipated from the LacZ staining pattern, we observed partial recombination of the Stat3−/− alleles and associated reduction in Stat3 expression in the tumors (Supplementary Fig. S1D and S1E). Importantly, 8 weeks later, we observed reduced tumor burden in the tamoxifen-treated cohort when compared with the vehicle control group, which coincided with a reduction in the tumor size, but not in the tumor number (Fig. 1B). Furthermore, in tamoxifen-treated gpa33-CreERT2gp130F/F;Stat3−/− mice, we observed reduced staining for the proliferation marker PCNA (proliferating cell nuclear antigen) in the dysplastic epithelium of the branching, tortuous gastric glands in the tumors that often develop cystic dilations (15). These histopathologic
observations correlated with reduced expression of the Stat3 target gene CcnD1 encoding the cell-cycle regulator cyclin D1 (Fig. 1C and 1D). Collectively, these data suggest that genetic suppression of Stat3 expression in neoplastic epithelium reduces tumor growth in the antrum of gp130F/F mice.

AZD1480 treatment reduces gastric tumor burden in gp130F/F mice

To test whether pharmacologic inhibition of Jak kinase activity would confer a therapeutic benefit in gp130F/F mice, we treated 8-week-old mice with AZD1480 for 6 weeks (Fig. 2A). In mice collected immediately after the end of this treatment period (referred to as "Acute cohort"), we observed a reduced tumor burden (Fig. 2B and Supplementary Fig. S2). Histologic analysis of lesions from the AZD1480-treated cohort revealed that they were entirely intramucosal and lacked the signs of hemorrhage and inflammatory cell infiltrates observed in tumors of untreated mice. The latter showed the previously observed mucus metaplasia with signs of epithelial invasion into muscularis and chronic abrasion of the epithelium facing the lumen of the stomach (Supplementary Fig. S2A and ref. 15). Likewise, mice that were allowed to live for 6 additional weeks after AZD1480-treatment ("Follow-up cohort") also had reduced tumor burden when compared with their age-matched vehicle cohort (Fig. 2B and Supplementary Fig. S2). However, the tumor burden in 20-week-old mice of the "Follow-up" cohort was larger than in the 14-week-old AZD1480-treated "Acute" cohort, indicating that the growth inhibitory effect of AZD1480 was reversible. The reduced number of both, emerging small (<25 mg) as well as established large tumors (25–50 mg), suggests that AZD1480 treatment suppressed early steps of tumorigenesis as well as subsequent tumor growth (Fig. 2B). Accordingly, in the "Follow-up" cohort, we observed an increase in larger tumors, presumably arising from the small (25–50 mg) tumors, as well as a "replenishment" of newly arising tumor in the latter group.

AZD1480 reduces gastric tumor burden in a Stat3-dependent manner

We next confirmed that AZD1480 inhibited Jak1/2 activity in the tumors by reduced abundance of phosphorylated Jak1 (pJak1) and Jak2 (pJak2), and this difference was no longer observed in the "Follow-up" cohort (Fig. 3A). We also performed immunohistochemical analysis for phosphorylated Stat3 (pStat3) to confirm that AZD1480 treatment suppressed Stat3 activity, and observed a marked reduction in pStat3 expression in
tumors from the "Acute" cohort (Fig. 3A and Supplementary Fig. S3A), which completely reverted in the "Follow-up" cohort. As predicted from the original application of AZD1480 to inhibit Jak2-mediated Stat5 activation in hemopoietic cells (7), we also detected reversible inhibition of Stat5 in these tumors most likely arising from tumor-infiltrating cells (13). Therefore, we investigated whether AZD1480 treatment also affected phosphoinositide 3-kinase (PI3K) pathway activation. Surprisingly, we observed similar staining for the mTOR expression following AZD1480 treatment (Fig. 3C). Collectively, these findings suggest that gp130-dependent activation of the PI3K/mTOR pathway may occur independent of Jak1/2 activity. The observation coincided with reduced expression of the cell-cycle regulators ccnd1 and cdc25 in the "Acute" cohort (Supplementary Fig. S3D). This finding was consistent with Western blot analysis that revealed reduced expression of the Stat3 target genes encoding the prosurvival proteins Bcl-XL and Bcl-2 in the "Acute," but not in the "Follow-up" cohort (Fig. 3A). Likewise, AZD1480 also reduced the number of PCNA-positive epithelial cells in tumors of the "Acute," but not of the "Follow-up" cohort (Supplementary Fig. S3D) and this observation coincided with reduced expression of the cell-cycle regulators ccd1 and cdc25 (Fig. 3D). Collectively, our data indicate that AZD1480 treatment reduces Stat3 activity and limits the growth of gastric tumors in gp130F/F mice by simultaneously suppressing cell survival and proliferation.

AZD1480 suppresses tumor growth in inflammation-associated colon cancer in wild-type mice

To investigate whether Jak1/2 inhibition could also confer therapeutic benefits in an inflammation-associated tumor model in which all gp130-signaling components remained wild-type, we explored the efficacy of AZD1480 treatment in the CAC model in the wild-type mice. The CAC model depends on the administration of the somatic
mutagen azoxymethane (AOM) to induce mutations in exon 3 of the *ctnnb1* gene to confer constitutive activation of the canonical WNT signaling pathway (3, 13). Subsequent repeated administration of the luminal irritant dextran sodium sulfate (DSS) in the meanwhile triggers flares of colonic inflammation and the development of colitis (refs. 3, 12, 13; Supplementary Fig. S4A). We and others have shown that in the CAC model, IL-6 family cytokines promote survival and proliferation of AOM-mutagenized cells to form tubular adenomas in the distal colon, which is suppressed in the absence of Stat3 expression in the intestinal epithelium (3). We therefore randomly assigned CAC-challenged mice after the second cycle of DSS to AZD1480 or control treatments and we used serial endoscopy before and at the end of the 40-day treatment period to monitor tumor progression in individual mice. By using established scoring parameters for the endoscopic examinations (11, 13), we detected the expected increase in disease in the vehicle-treated cohort. In contrast, the disease scores remained stable or decreased in all mice of the AZD1480-treated cohort (Fig. 4A and Supplementary Fig. S4B). Furthermore, histologic analyses of colonic tissue revealed a reduction in PCNA-positive epithelial staining in colons of AZD1480-treated mice, which coincided with a reduction of inflammatory cell aggregates (Supplementary Fig. S4C). Western blot analysis revealed that the colonic epithelium of AZD1480-treated mice contained less pJak1 and pJak2, which correlated with suppressed pStat3 (and pStat5) levels when compared with the colonic epithelium of the control cohort. Likewise, we also observed reduced abundance of Bcl-2 and Bcl-XL proteins in colonic epithelium of AZD1480-treated mice (Fig. 4C) although the abundance of IL-11 protein in these tumors remained unaffected. Collectively, these data indicate that AZD1480 treatment effectively suppressed the tumor growth in wild-type mice and that the underlying molecular mechanism is likely to relate to the impairment of Stat3-driven proliferation and survival of neoplastic intestinal epithelium.

**Discussion**

The tumor microenvironment is a rich source of inflammatory cytokines, among which the IL-6 family is most widely implicated in conferring tumor-promoting activities in breast, prostate, liver, and gastrointestinal cancers (2). In turn, persistent and aberrant activation of Stat3 in

![Figure 3. Biochemical analysis of gastric tumors from AZD1480-treated gp130F/F mice.](image-url)
neoplastic tissue promotes expression of many hallmarks of cancer (16) and is associated with a poor prognosis in many solid cancers including those arising from gastric (17) and colonic epithelium (18). In human cancers, somatic mutations have been identified that confer constitutive activation of GP130, JAK1, JAK2, or STAT3 alongside epigenetic silencing of the negative regulator SOCS3. However, in the majority of cases excessive signaling results from an overabundance of IL-6 family ligands (2). Here, we show in two independent models of inflammation-associated cancer that the dual Jak1/2 inhibitor AZD1480 confers therapeutic benefits.

In both models utilized here, the inhibition of tumorigenesis coincided with reduced Stat3 phosphorylation, reduced expression of the antiapoptotic proteins Bcl-XL and Bcl-2, and the cell-cycle regulators Ccnd1 and Cdc25. These observations are consistent with findings in xenograft models in which Jak1/2 inhibition reduces the growth of breast, prostate, and ovarian cancers and coincided with reduced pStat3 expression in glioblastoma (19), thyroid carcinoma (20), and myeloma (21). We have previously shown that epithelial-specific Stat3 deletion in the intestine confers a prophylactic benefit to CAC-challenged mice (3). Here, we observe that mosaic lacZ activation by gpa33-CreERT2 and the corresponding reduction of Stat3 expression by approximately 30% is sufficient to reduce overall tumor growth. It is therefore likely that at least some of the effects conferred by systemic AZD1480 administration occurred at the level of the neoplastic epithelium. These findings support an exquisite sensitivity of tumor cells to excessive Stat3 signaling in which, for example, genetic ablation of one Stat3 allele is already sufficient to block gastric tumorigenesis in gp130F/F; Stat3+/−/C0 mice (8). By the same token, collectively, our above observations argue strongly against a significant contribution of Stat5 inhibition, which we also observed in AZD1480-treated mice. Conversely, normal tissue homeostasis remains unaffected in Stat3+/−/C0 mice (13) and is also not affected after a 40-day treatment with AZD1480 (Stuart and colleagues; unpublished data). However, systemic Jak-inhibition may also confer additional therapeutic benefits, including suppression of tumor angiogenesis and possibly restore the antitumor response frequently impaired as a consequence of excessive Stat3 activation in immune cells in response to tumor-derived production of cytokines such as IL-10 (22).

Developing therapeutic strategies for selective Stat3 inhibition has proven challenging and therefore targeting

Figure 4. Systemic administration of AZD1480 reduces established CAC burden in wild-type mice. A, tumor burden of individual mice was serially assessed by endoscopy as described (11, 13), immediately before and at the end of a 40-day treatment of AZD1480 (30 mg/kg, per os once daily for 5 days/week) or vehicle. Mice were randomly assigned to the AZD1480 and vehicle treatment control cohorts. Resulting endoscopy scores for each cohort were statistically assessed. B, total number of macroscopically visible colon tumors per mouse at time of autopsy. The combined tumor area was estimated from assuming a circular shape of the resulting adenomas and determining their diameter. n ≥ 3 mice per cohort. C, representative Western blot analysis for the indicated proteins and their phosphorylated (p) isoforms of cell lysates prepared from pooled colonic tumors of individual mice collected at the end of a 40-day treatment of AZD1480 (30 mg/kg, per os once daily for 5 days/week) or vehicle. Data are mean ± SEM with *, P < 0.05.
of upstream components provides a pharmacologically viable alternative including monoclonal antibodies directed toward IL-6 or its α-chain receptor subunit or small-molecule inhibitors for Jak family of kinases. Although originally developed to inhibit the constitutive active JAK2(V617F) mutant associated with myeloproliferative disorders (7), here we show surprising efficacy of AZD1480 on excessively activated wild-type Jak1 and Jak2 to confer a cytostatic effect. Because recent evidence suggests that combinations of Jak inhibitors with histone deacetylase (HDAC) and mTOR antagonists provide an effective strategy to induce killing of tumor cells harboring the JAK2(V617F) mutations (23) similar combinations may result in the desired killing of cancerous gastrointestinal epithelium.

Disclosure of Potential Conflicts of Interest

D. Huszar has ownership interest (including patents) in AstraZeneca. No potential conflicts of interest were disclosed by the other authors.

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Therapeutic Inhibition of Jak Activity Inhibits Progression of Gastrointestinal Tumors in Mice

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