Selective inhibition of inflammatory gene expression in activated T lymphocytes: a mechanism of immune suppression by thiopurines


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ABSTRACT

Azathioprine and 6-mercaptopurine are anti-metabolite thiopurine drugs that play important roles in the treatment of leukemia, and in the management of conditions requiring immunosuppression, such as inflammatory bowel disease. The biochemical pharmacology of these drugs suggests that inhibition of purine nucleotide formation through the 6-thioguanine nucleotide metabolites is their key molecular mechanism. However, it is unclear how these metabolites suppress immunity. We hypothesized that azathioprine produces a selective inhibitory effect on activated but not quiescent T lymphocytes. We first established a model system of T lymphocyte culture with azathioprine that produced pharmacologically relevant concentrations of 6-thioguanine nucleotides. Using genome-wide expression profiling, we identified a group of azathioprine-regulated genes in quiescent and activated T lymphocytes. Several genes involved in immunity and inflammation were selectively down-regulated by azathioprine in stimulated but not quiescent cells. Quantitative RT-PCR for 3 of these genes, tumor necrosis factor-related apoptosis-inducing ligand, tumor necrosis factor receptor superfamily member 7 and α4-integrin, confirmed down-regulated expression of transcript levels. Tumor necrosis factor-related apoptosis-inducing ligand protein expression was further studied and found to be inhibited by azathioprine, 6-mercaptopurine and 6-thioguanine, implying that the inhibitory effects of azathioprine on expression are mediated by 6-thioguanine nucleotides. These results therefore provide a previously unrecognized molecular mechanism for the immunosuppressive properties of thiopurine anti-metabolite drugs.
The anti-metabolite thiopurine medications, azathioprine (AZA, 6-(1-Methyl-4-nitroimidazol-5-yl)thiopurine) and 6-mercaptopurine (6-MP), are important therapies for both induction and maintenance of remission in patients with Crohn’s disease and ulcerative colitis (Pearson et al., 1995; Egan and Sandborn, 2004). Although originally developed for use in leukemia, AZA and 6-MP are now commonly used for their immunosuppressive properties in solid organ transplantation, autoimmune hepatitis, rheumatoid arthritis and autoimmune dermatologic diseases in addition to inflammatory bowel disease (Lake et al., 2000). Once ingested, these drugs enter a metabolic pathway that is complex and produces several different final products. AZA is non-enzymatically converted to 6-MP, which can be metabolized to the inactive metabolites 6-thiouric acid via xanthine oxidase, or 6-methylmercaptopurine via thiopurine methyltransferase. 6-Thioguanine nucleotides (6-TGN), which are active metabolites of AZA and 6-MP, are produced through the sequential actions of hypoxanthine phosphoribosyl transferase, inosine monophosphate dehydrogenase and guanosine monophosphate synthetase (Aarbakke et al., 1997). In lymphocytes, this results in the depletion of purine nucleotides, especially those containing adenosine (Dayton et al., 1992). As a consequence of the lack of purine nucleotides, 6-TGN inhibit the synthesis and utilization of precursors of DNA, effects which probably underlie the anti-proliferative actions of AZA and 6-MP. However, in addition to the formation of precursors of DNA, purine nucleotides are also essential in RNA synthesis, and in phosphorylation and glycosylation reactions. This complexity raises the possibility that thiopurine drugs might have pleiotropic effects in cells, in addition to inhibiting proliferation. Currently, if remains unclear if inhibition of lymphocyte proliferation is a relevant mechanism underpinning
the immunosuppressive effect of thiopurine drugs. Indeed, overt inhibition of lymphocyte numbers in circulating blood is not required for the therapeutic effects of AZA or 6-MP, but is considered an adverse effect that correlates with the occurrence of opportunistic infections (Connell et al., 1993).

Since the therapeutic immunosuppression of AZA and 6-MP is evident in the setting of chronic inflammatory diseases, but usually does not predispose to opportunistic infections, we reasoned that these drugs act by selectively affecting activated but not resting immune cells. To test this possibility, we developed an in vitro system of AZA-treated T lymphocytes that models closely the effects of AZA therapy on those cells in vivo. Using a genome-wide expression profiling approach, we evaluated the effects of AZA on the expression of genes in both resting and stimulated T lymphocytes. Our results have identified several immune and inflammation-related genes whose upregulated expression is potently inhibited by thiopurines in activated but not resting T lymphocytes, suggesting potential novel immunosuppressive mechanisms of these drugs.
METHODS

Reagents:

Unless otherwise stated, reagents were obtained from Sigma-Aldrich Chemical Company (St. Louis, MO)

Cell Culture:

Ju.1 T lymphocytes which stably express the interleukin (IL)-1 receptor were used for these studies (McKean et al., 1995). Cells were cultured in RPMI supplemented with 5% fetal calf serum and 2mmol/l L-glutamine (Invitrogen, Carlsbad, CA). For experiments, log-growth phase cells were cultured in medium supplemented with varying concentration of AZA, 6-MP or 6-TG. Cells were stimulated with 5µg/ml cross-linked OKT3 (Janssen, Titusville, NJ) plus 0.25 ng/ml recombinant human IL-1α, as previously described (McKean et al., 1995; Kalli et al., 1998). In some experiments, cells were stimulated using plate-bound OKT3 alone, prepared by incubation of plastic tissue culture plates with a 0.5 µg/ml solution of OKT3 before addition of cells, or in combination with IL-1 or 1:20,000 diluted monoclonal antibody ascites 9.3 anti-CD28 (a gift of Carl June, University of Pennsylvania), which were added to culture medium.

Annexin V and propidium iodide staining:

Cells were collected, washed with cold PBS and re-suspended in tubes containing FITC-Annexin V (Beckman-Coulter, Hialeah, FL). Cells were incubated for 20 minutes at 4°C and then were washed before resuspension in buffer containing propidium iodide (0.5 µg/mL). Flow cytometry was performed and analyzed using FACS-Calibur and
CellQuest (Becton Dickinson). Apoptotic cells were Annexin V positive but propidium iodide negative.

**Human Peripheral Blood T Lymphocyte Studies:**

After obtaining approval from the Institutional Review Board of the Mayo Foundation, 6 inflammatory bowel disease patients taking AZA and 2 taking 6-MP provided 50 ml blood samples. The mean dose of AZA was 2.1 mg/kg/day for a mean duration of 30 months and the mean dose of 6-MP was 1.15 mg/kg/day for a mean duration of 45 months. Fifty ml of whole blood was divided into two 50ml conical tubes. Mononuclear cells were extracted from whole blood using Isolymph (Gallard-Schlesinger, Plainview, NY), according to manufacturer’s instructions. Phagocytic cells were removed by L-leucine methyl ester incubation and then cells were filtered through nylon mesh to remove platelet clumps. T cell rosetting was performed using sheep RBCs (Colorado serum, Denver, CO) and ammonium chloride potassium lysis buffer (Cambrex, East Rutherford, NJ). The isolated T lymphocytes were re-suspended in RPMI culture medium supplemented with 10% fetal calf serum, and under these purification procedures have been found to contain less than 5% cells other than T lymphocytes.

**6-TGN assay:**

Intacellular 6-TGN concentration was measured as described previously (Pike et al., 2001).
Sample preparation and scanning for Affymetrix Gene Chip Expression Profiling:

Total RNA was isolated from Ju.1 cells and purified using commercial affinity resin column kits (Qiagen, Chatsworth, CA). cDNA was prepared from a total of 10 µg RNA pooled from 3 independent experiments to minimize the effect of random variation on the expression profiles. The purified cDNA was used as a template for in vitro transcription reaction for the synthesis of biotinylated cRNA using RNA transcript labeling reagent (Affymetrix, Santa Clara, CA). Labeled cRNAs were then fragmented and hybridized onto the U133A array. Appropriate amounts of fragmented cRNA and control oligonucleotide B2 were added along with control cRNA (BioB, BioC, BioD), herring sperm DNA and bovine serum albumin to the hybridization buffer. The hybridization mixture was heated at 99 °C for 5 minutes followed by incubation at 45 °C for 5 minutes before injecting the sample into the microarray. Then the hybridization was carried out at 45 °C for 16 h with mixing on a rotisserie at 60 r.p.m. Following hybridization, the solutions were removed, the arrays were washed and then stained with streptavidin-phycoerythrin (Molecular Probes, Eugene, OR). Following washes, probe arrays were scanned using the Genechip system confocal scanner (Hewlett-Packard, Palo Alto, CA). The quality of the fragmented biotin labeled cRNA in each experiment was evaluated before hybridizing onto the U133A expression array by both gel electrophoresis and hybridizing a fraction of the sample onto test-3 array as a measure of quality control.

Gene Chip Data analysis:
GeneChip 5.0 (Affymetrix, Santa Clara, CA) was used to scan and quantitatively analyze the scanned image. Once the probe array has been scanned, GeneChip software automatically calculates intensity values for each probe cell and makes a presence or absence call for each mRNA. Algorithms in the software use probe cell intensities to calculate an average intensity for each set of probe pairs representing a gene, which directly correlates with the amount of mRNA. Spotfire (Spotfire, Cambridge, MA) and Microsoft Excel are also used for data analysis. Expression patterns for each group were compared with the control group. When assessing the difference between two different RNA samples, the fold changes from side-by-side experiments on the same lot of microarrays were compared directly. In this analysis, we considered gene transcripts altered $\geq$2-fold as significant. Gene expression profiles were analyzed at the Mayo General Clinical Research Center Genomics, Proteomics and Metabolic Core Facility using these established protocols (Sreekumar et al., 2002a; Sreekumar et al., 2002b).

**Real-time reverse transcription (RT)-PCR:**

cDNA was prepared using Superscript II (Invitrogen). Sequence specific RT-PCR primers were designed using Affymetrix and Primer 3 algorithms (sequences available on request). Real-time PCR reactions were carried out using SYBR Green master mix on a 7700 Sequence Detector instrument (Applied Biosystems, Foster City, CA). Relative mRNA expression between samples was quantified using the $\Delta\Delta$Ct method where $\beta$-actin mRNA was utilized as an internal control, as previously described (Egan et al., 2004).
**TRAIL and IL-2 ELISAs:**

A human tumor necrosis factor (TNF) related apoptosis-inducing ligand (TRAIL) ELISA kit (Biosource International, Camarillo, CA), and a human IL (IL)-2 ELISA kit (BD Pharmingen, San Diego, CA) were used to quantify expression of those proteins, using the manufacturers’ instructions. Microtiter plate reading of experimental samples and purified standards was performed at 450nm. The BCA protein assay (Pierce, Rockford, IL) was used to quantify protein concentration for normalization of TRAIL and IL-2 expression.
RESULTS

AZA-treated Ju.1 T cells: an in vitro model of AZA therapy

Most studies of 6-TGN in patients receiving chronic thiopurine therapy have measured concentrations of this metabolite in erythrocytes, but not in lymphocytes, the likely target cells. Therefore, in preliminary experiments, we initially measured the concentration of 6-TGN in lymphocytes and in whole blood from 8 inflammatory bowel disease patients receiving stable doses of AZA or 6-MP (Figure 1). We found that the mean 6-TGN concentration in lymphocytes of 5590 pmol/8 x 10^8 cells was 26-fold higher than the mean whole blood concentration, consistent with the notion of 6-TGN incorporation into DNA in target cells.

Next, we developed a model system in vitro of culturing Ju.1 cells with varying concentrations of AZA for varying time periods and assessed the accumulation of intracellular 6-TGN. We found that 6-TGN accumulated in cells in an AZA dose-dependent fashion (Figure 2A). After 48 hours of culture in AZA at concentrations from 1 to 10 µmol/l, Ju.1 cells contained 6-TGN in the concentration range of approximately 3,000 to 30,000 pmol/8 x 10^8 cells. While AZA 1 µmol/l did not significantly affect Ju.1 cell growth at 48 hours, AZA 10 µmol/l caused an approximately 70% reduction in Ju.1 cell number at this time (not shown). Based on these data, we treated Ju.1 cells with AZA 1 µmol/l for 48 hours for expression profiling experiments, since this concentration produced 6-TGN concentrations in cells that approximated pharmacologically observed values in relevant target cells in vivo, and did not overtly inhibit cell proliferation.

Additional experiments established that incubation with AZA at concentrations up to 10 µmol/l for 48 hours did not induce significant apoptosis or death in Ju.1 cells, judged by
Annexin V (Figure 2B) and propidium iodide staining. To model T lymphocyte activation, Ju.1 T lymphocytes were left unstimulated or stimulated by T cell receptor crosslinking, utilizing biotinylated OKT3 in the presence of streptavidin, plus IL-1. Prior studies had established that such stimulation resulted in robust activation of Ju.1 cells as assessed by nuclear factor-κB activity, and IL-2 expression (McKean et al., 1995; Kalli et al., 1998).

**Microarray Results: AZA affects the expression of a subset of genes in Ju.1 T lymphocytes**

For expression profiling experiments, Ju.1 cells were cultured until in exponential phase of growth and then divided into 4 subcultures. Two control subcultures were maintained in standard growth medium, and in 2 others, AZA 1 μmol/l was added to standard growth medium. After 48 hours, 1 control and 1 AZA-treated subculture were stimulated with OKT3 plus IL-1, while the other subcultures were left unstimulated. 24 hours later, cells were collected, washed and RNA was extracted. This experiment was conducted on 3 independent occasions, and equal amounts of RNA were pooled from the 3 experiments for each condition. The pooled RNA was then processed for gene chip hybridization.

Table 1 lists the gene whose expression was significantly (> 2-fold) affected by AZA in unstimulated Ju.1 cells. Six genes, mainly involved in metabolism, were downregulated by AZA treatment in unstimulated Ju.1 cells. Twenty genes were significantly upregulated by AZA treatment in unstimulated cells, and were mainly involved in metabolism and signal transduction. Table 2 lists the gene whose expression
was significantly (> 2-fold) affected by AZA in Ju.1 cells that were stimulated with
OKT3 plus IL-1. We identified 11 genes that were significantly downregulated by AZA
treatment. Most of these genes were involved in immune functions. In 8 of these 11 genes
downregulated by AZA, stimulation had induced an upregulation of expression. Fifteen
genes were upregulated by AZA in stimulated Ju.1 cells, and most of these were involved
in metabolism. The effect of stimulation on these genes was varied.

**Real time RT-PCR analysis of candidate gene expression**

Gene chip studies identified many genes whose expression was affected by AZA,
including some with prominent immune and inflammatory functions. From the lists of
genes in tables 1 and 2, we chose for further study by real-time RT-PCR, 2 genes whose
upregulated expression was dampened by AZA in stimulated Ju.1 cells, TNF ligand
superfamily member 10, also known as TRAIL, and TNF receptor superfamily member 7
(TNFRSF7). We also further studied $\alpha_4$-integrin, a gene whose expression was
downregulated by AZA in unstimulated Ju.1 cells. Expression of TRAIL mRNA after
Ju.1 cell stimulation increased by 32.9 fold compared to unstimulated cells (Figure 3A).
In the presence of 10 $\mu$mol/l AZA, the expression of TRAIL was blunted to an increase
of 10.7 fold by stimulation. A similar phenomenon was noted in TNFRSF7 expression
whereby a 27.7 fold increase was noted after stimulation, which was blunted in the
presence of 10 $\mu$mol/l AZA to 12.8 fold. Stimulation of Ju.1 cells had little effect on the
expression of $\alpha_4$ integrin, but a significant 6-fold down-regulation in $\alpha_4$ integrin
expression was noted in the 10 $\mu$mol/l AZA treated stimulated cells compared to
untreated stimulated cells. In contrast to results obtained from the gene chip, by real-time
RT-PCR, AZA did not significantly reduce α4-integrin mRNA expression in unstimulated cells, but did in stimulated cells. We further evaluated the effect of varying concentrations of AZA on the expression of TRAIL, TNFRSF7 and α4-integrin in stimulated Ju.1 cells. A dose-dependent inhibition of expression of these 3 genes was observed (Figure 3B). Together, these results generally confirmed and validated the data obtained from the gene chip expression studies. Interestingly, when analyzed by the more quantitative real-time RT-PCR approach, it is clear that the gene chip underestimated the magnitude of the differential expression of these genes.

**Effect of AZA on TRAIL protein expression**

For further studies, we focused on TRAIL, since this cytokine shares many similarities with the pro-inflammatory cytokine TNF-α, and plays important roles in immune and inflammatory functions (Smyth et al., 2003). We used a specific ELISA to quantify the effect of AZA on TRAIL protein production in Ju.1 cells. Stimulation with OKT3 plus IL-1 resulted in marked upregulation of TRAIL expression, which could be blocked by AZA (Figure 4A). AZA alone did not affect TRAIL expression, but produced a dose-dependent inhibition of stimulated TRAIL expression in Ju.1 cells (Figure 4B). The inhibitory effect of AZA on TRAIL expression was progressive with increasing durations of pre-treatment with AZA prior to stimulation, up to 48 hours (Figure 4C). This is consistent with the time-dependent accumulation of 6-TGN in AZA-treated cells. To extend the observations in Ju.1 cells, we also evaluated the effect of AZA on TRAIL expression in primary T lymphocytes. TRAIL was not detected in unstimulated primary T lymphocytes. Consistent with Ju.1 cell results, we observed a dramatic upregulation of
TRAIL expression by stimulation of primary T lymphocytes with OKT3 plus IL-1. AZA treatment was effective in partially blocking the upregulated expression of TRAIL by stimulation (Figure 4D), a finding which supports the importance of results obtained in Ju.1 cells.

**AZA selectively abrogates Ju.1 cell TRAIL expression irrespective of co-stimulatory signal.**

The activation of T lymphocytes requires a signal from the ligated T cell receptor, which is usually greatly amplified by a second, or co-stimulatory signal. To determine if the effect of AZA on TRAIL expression in Ju.1 cells was sensitive to the co-stimulatory stimulus, we measured TRAIL production under varying conditions of co-stimulation. The addition of either soluble IL-1 or anti-CD28, both established T lymphocyte co-stimulatory signals, to a T cell receptor stimulus in the form of plate-bound OKT3, greatly increased TRAIL production in Ju.1 cells (Figure 5A). AZA almost completely abolished TRAIL expression under all of these conditions. In contrast, AZA had only a relatively minor, although statistically significant effect on IL-2 secretion by Ju.1 cells under the same conditions (Figure 5B). These results indicate that AZA blocks TRAIL expression induced by T cell activation under a variety of stimulation conditions, and further show the specificity of the effect since Ju.1 cells, despite almost complete shut-down of TRAIL expression, can still mount a robust IL-2 response to stimulation.
**6-MP and 6-TG (2-Amino-6-mercaptopurine) inhibit TRAIL expression in stimulated Ju.1 cells**

Current concepts of thiopurine anti-metabolite drugs’ mechanism of immunosuppression invoke 6-TGN as common effector entities that affect the target cells. We therefore reasoned that 6-MP and 6-TG would produce effects on TRAIL production similar to what we observed with AZA. To assess this possibility, we pre-treated Ju.1 cells with varying concentrations of 6-MP or 6-TG, and measured the expression of TRAIL after stimulation with OKT3 plus IL-1. We observed a dose-dependent inhibition by both 6-MP and 6-TG, of TRAIL expression in stimulated cells (Figure 6). This strongly suggests that the inhibitory effect of thiopurines on inflammation-induced gene expression in T lymphocytes depends on 6-TGN metabolic products.
DISCUSSION

The mechanism of action of AZA has remained poorly understood despite decades of use in the treatment of malignancy and conditions requiring immunosuppression. We hypothesized that the immunosuppression caused by AZA results from selective effects on the expression of specific genes in activated lymphocytes. Using a genome-wide expression profiling approach, we identified a number of candidate genes whose upregulated expression in T lymphocytes was inhibited by AZA. Those genes identified with this approach included some with prominent immune and inflammatory functions, such as α4-integrin, TNFRSF7 and TRAIL. We further established that, in the case of TRAIL, 6-MP and 6-TG produced similar inhibitory effects on expression. This indicates that thiopurine anti-metabolite drugs such as AZA, selectively inhibit the expression of immune and inflammation-related genes in activated T lymphocytes, effects which are likely mediated through 6-TGN. These findings provide a previously unrecognized molecular mechanism of immunosuppression by AZA, which might underlie the beneficial actions of thiopurines in inflammatory disease states.

Through a complex metabolic pathway (Aarbakke et al., 1997), thiopurine drugs lead to the intracellular accumulation of 6-TGNs, which interfere with the synthesis of DNA, affect the expression of certain genes, and inhibit cell proliferation. For these reasons, 6-TGNs are likely to be the active metabolites of thiopurines that mediate their therapeutic effects. In some studies, circulating 6-TGN levels have been proposed for therapeutic drug monitoring during thiopurine treatment of inflammatory bowel disease (Cuffari et al., 1996b; Dubinsky et al., 2000; Cuffari et al., 2001; Dubinsky, 2003). This is based on the
observation that 6-TGN concentration in erythrocytes correlates with the dose of AZA or 6-MP, and in some series 6-TGN correlates with clinical response to these drugs. However, the clinical utility of measuring 6-TGN levels in inflammatory bowel disease patients has been challenged by studies that found no correlation with clinical response when thiopurine dose had been adjusted for thiopurine methyltransferase activity (Lowry et al., 2001).

Nevertheless, the importance of 6-TGN is further supported by the apparent clinical benefit of 6-TG therapy in inflammatory bowel disease patients (Dubinsky et al., 2003a). Unfortunately, 6-TG appears to cause significant hepatotoxicity, seriously limiting its clinical utility (Dubinsky et al., 2003b).

While most studies have measured the concentration of 6-TGN in erythrocytes since these cells are easily obtained, we wished to measure 6-TGN concentration in relevant target cells. We therefore purified lymphocytes from peripheral blood of inflammatory bowel disease patients receiving standard doses of AZA or 6-MP and measured 6-TGN in those cells and in erythrocytes. We found a much higher concentration in lymphocytes, which probably reflects incorporation of 6-TGN into cellular DNA. Our results of 6-TGN accumulation in leukocytes are consistent with prior reports (Cuffari et al., 1996a).

We utilized a genome-wide expression-profiling approach to identify genes in a T lymphocyte model, whose up-regulated expression was inhibited by AZA at concentrations that are pharmacologically relevant and result in 6-TGN levels that are seen in vivo. Using this approach, we identified 8 genes whose upregulated expression was inhibited more than 2-fold by AZA. Among these AZA-regulated genes were 2 that are known to possess important immune and inflammation-related functions, TRAIL and TNFRSF7. TRAIL, also known as Apo-2 ligand is a member of the TNF superfamily of cytokines. TRAIL is
expressed widely within the immune system, especially on lymphocytes, and its production is induced by many stimuli that are associated with inflammation, in part through the action of transcription factor nuclear factor κB (Rivera-Walsh et al., 2001). Similar to TNF, TRAIL is expressed by many cells in a membrane-bound form, or it can be secreted. Many cells possess plasma membrane receptors for TRAIL, of which there are at least 5 distinct forms. Only DR4 ligation by TRAIL results in transmission of a signal into cells, the other receptors lacking known functions, other than potentially acting as “decoys” to buffer the effects of TRAIL (Sheridan et al., 1997). TRAIL, acting through DR4, initiates caspase activation that results in apoptosis, primarily of transformed cells (Smyth et al., 2003). Considering the established potential for AZA and 6-MP to induce non-Hodgkins lymphomas, it is intriguing to speculate that inhibition of TRAIL expression, an important inducer of apoptosis in transformed lymphocytes, might represent a molecular mechanism for lymphomagenesis in thiopurine-treated patients. The actions of TRAIL on non-transformed cells include induction of nuclear factor κB, which tends to counterbalance caspase activation, in a manner similar to that observed with TNF. Recent reports have highlighted the function of plasma membrane-associated TRAIL as a receptor for DR4. In this model, TRAIL transmits a signal into the cells on which it is expressed, for example lymphocytes, when ligated by DR4 that is expressed on other cells (Chou et al., 2001). In lymphocytes, this results in proliferation and interferon-γ secretion, markers of activation. Thiopurine-mediated interference in such a mechanism could dampen over-active immune responses, such as occur in inflammatory bowel disease.
We also identified TNFRSF7 (CD27) as a gene upregulated by T lymphocyte activation that is significantly inhibited by AZA. TNFRSF7 is member of the TNF receptor superfamily that is involved in the regulation of T lymphocyte activation, as assessed by nuclear factor κB activation and interferon-γ secretion (Yamamoto et al., 1998). Recent evidence suggests that T lymphocyte activation after ligation of the T cell receptor can be significantly augmented by signaling from TNFRSF7 when this receptor is activated by its ligand CD70 (Prasad et al., 1997; Hendriks et al., 2000). Along with the inhibitory effect on TRAIL expression, down-regulation of TNFRSF7 by AZA would decrease the responsiveness of T lymphocytes to activation, an immunomodulating effect consistent with this drugs clinical effects.

The expression profiling studies also identified α4-integrin as an AZA-regulated gene. T lymphocyte stimulation had little effect on the expression levels of α4-integrin, but AZA resulted in a significant down-regulation of it’s expression on quiescent cells. While real time RT-PCR studies did not confirm lower expression of α4-integrin in AZA-treated unstimulated cells, we did observe significantly lower expression of this gene in AZA-treated cells after stimulation. These discrepancies highlight the need to confirm gene chip data with an independent technique. We chose α4-integrin for further study because of compelling recent evidence that implicates this adhesion molecule in the pathogenesis of 2 important immunological diseases, Crohn’s disease and multiple sclerosis (Ghosh et al., 2003; Miller et al., 2003). α4-integrin is one component of heterodimeric adhesion molecules that are expressed on the surface of activated lymphocytes and monocytes (von Andrian and Engelhardt, 2003). α4-integrin, along with one of two beta subunits, interacts with cognate selectin ligands expressed on endothelium, thereby mediating efflux of
activated lymphocytes and monocytes from the vasculature into sites of inflammation (Berlin et al., 1995). Downregulated expression of α4-integrin by AZA might therefore act to inhibit the emigration of T lymphocytes, or monocytes, from blood vessels at sites of inflammation. Such an effect on leukocyte recruitment would serve to decrease injury and inflammation, potentially relevant mechanisms.

A number of recent reports have highlighted the ability of thiopurines to induce apoptosis of activated T lymphocytes. Such an effect could rationally result not only in the anti-neoplastic effects of AZA and 6-MP, but also in immunosuppression since it might reduce the numbers of activated cells causing disease. One report showed that 6-MP could block cell cycle progression of T lymphocytes, and could induce apoptosis, but only if the cells were actively proliferating (Quemeneur et al., 2003). In another recent publication, AZA or 6-MP, acting through their 6-thio-GTP metabolites, were found to block Rac1 (Tiede et al., 2003). In activated lymphocytes, this resulted in apoptosis, but only after prolonged incubation over several days. Our results point towards an alternative anti-inflammatory or immunosuppressive effect of thiopurine drugs, in which the stimulus-induced expression of specific genes by T lymphocytes is greatly inhibited. AZA-treated Ju.1 cells did not undergo apoptosis significantly more than control cells under the conditions of our experiments. However, our results are not inconsistent with those of Quemeneur (Quemeneur et al., 2003) and Tiede (Tiede et al., 2003) since we utilized different cells, different concentrations of drugs and shorter durations of incubation.

Based on our studies, a model of the immunosuppressive properties of thiopurines is presented in Figure 7. AZA or 6-MP therapy results in accumulation of 6-TGN in
lymphocytes. In the presence of T cell activation, as occurs in immunologically-driven diseases, 6-TGN blocks the expression of TRAIL, TNFRS7 and α4-integrin, effects which functionally decrease inflammation. These findings provide a new framework for understanding thiopurine-mediated immunosuppression, and further studies will establish the specific roles of these gene products in mediating the beneficial effects of AZA and 6-MP in immune diseases.
References


FOOTNOTES

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FIGURE LEGENDS

1. Whole blood and lymphocyte 6-TGN concentrations in 8 patients receiving stable doses of AZA or 6-MP.

2. A. Dose-dependent accumulation of 6-TGN in Ju.1 cells cultured with AZA. Cells were cultured in medium supplemented with the indicated amounts of AZA for 48 hours. 6-TGN were not detected in cells not cultured with AZA. Results are shown as mean + standard error. ANOVA, P < 0.05. B. Effect of AZA on apoptosis in Ju.1 cells. Cells were cultured in medium supplemented with the indicated amounts of AZA for 48 hours. Apoptosis was quantified by Annexin V and propidium iodide staining. Results are shown as mean + standard error. ANOVA, P > 0.05.

3. A. mRNA expression of TRAIL, TNFRSF7 and α4-integrin. Ju.1 cells were cultured in normal medium or in medium containing AZA 10 µmol/l for 48. Cells were then left unstimulated or were stimulated with OKT3 plus IL-1. After a further 12 hours, mRNA was extracted from cells and analyzed for the indicated targets by real-time RT-PCR. Results are expressed as (mean plus standard error) mRNA abundance relative to control untreated, unstimulated cells. Effect of AZA, t test * P < 0.05. B. Dose-dependent inhibition of TRAIL, TNFRSF7 and α4-integrin by AZA in stimulated Ju.1 cells. Ju.1 cells were cultured in normal medium or in medium containing the indicated concentrations of AZA for 48. Cells were then stimulated with OKT3 plus IL-1. After a further 12 hours, mRNA was extracted from cells and analyzed for the indicated targets by real-time RT-
PCR. Results are expressed as (mean plus standard error) mRNA abundance relative to control untreated stimulated cells. ANOVA, $P < 0.05$, all 3 genes.

4. A. Effect of AZA on TRAIL expression. Ju.1 cells were cultured in normal medium or in medium containing AZA 10 µmol/l for 48 hours. Cells were then left unstimulated or were stimulated with OKT3 plus IL-1. After a further 12 hours, TRAIL protein was quantified in cell lysates using a specific ELISA. Results are expressed as means plus standard error. Effect of AZA, $t$ test *$P < 0.05$. B. Time-dependent inhibition of stimulated TRAIL expression by AZA. Ju.1 cells were cultured in normal medium or in medium containing AZA 10 µmol/l for the indicated times. Cells were then stimulated with OKT3 plus IL-1 and after a further 12 hours, TRAIL protein was quantified in cell lysates using a specific ELISA. Results are expressed as means plus standard error. ANOVA, $P < 0.05$. ND, not detected. C. Dose-dependent inhibition of TRAIL expression by AZA. Ju.1 cells were cultured in normal medium or in medium containing the indicated concentrations of AZA for 48. Cells were then stimulated with OKT3 plus IL-1. After a further 12 hours, TRAIL protein was quantified in cell lysates using a specific ELISA. Results are expressed as means plus standard error. ANOVA, $P < 0.05$. D. Effect of AZA on TRAIL expression in primary T lymphocytes. T lymphocytes were extracted from blood and were cultured for 48 hours in normal medium or in medium containing AZA 10 µmol/l. Cells were then left unstimulated or were stimulated with OKT3 plus IL-1. After a further 12 hours, TRAIL protein was quantified in cell lysates using a specific ELISA.
Results represent a single determination of TRAIL at each point. ND, not detected.

5. Effect of AZA on TRAIL (A) and IL-2 (B) under different conditions of Ju.1 cell stimulation. Ju.1 cells were cultured in normal medium or in medium containing AZA for 48 hours. Cells were then left unstimulated or were stimulated with OKT3 alone, OKT3 plus IL-1 or OKT3 plus anti-CD28. After a further 12 hours, TRAIL protein was quantified in cell lysates, or IL-2 in culture medium, using specific ELISAs. Results are expressed as means plus standard error. Effect of AZA, t test *P < 0.05.

6. A. Dose-dependent inhibition of TRAIL expression by 6-MP (A) and 6-TG (B). Ju.1 cells were cultured in normal medium or in medium containing the indicated concentrations of drug for 48 hours. Cells were then stimulated with OKT3 plus IL-1. After a further 12 hours, TRAIL protein was quantified in cell lysates using a specific ELISA. Results are expressed as means plus standard error. ANOVA, P < 0.05, both drugs.

7. Proposed model for inhibition of T lymphocyte activation by thiopurines. AZA is non-enzymatically converted to 6-MP, which can be inactivated by thiopurine methyltransferase (TMPT) or xanthine oxidase (XO). 6-MP can also be metabolized by hypoxanthine phosphoribosyl transferase (HPRT), entering a metabolic pathway that produces 6-TGN, the active metabolite. 6-TG therapy also leads to production of 6-TGN which decreases synthesis of purine nucleotides. In T lymphocytes, this impairs the induced expression of pro-inflammatory TRAIL, TNFRSF7, α4-integrin and other genes upon activation of those cells.
Table 1. Genes significantly affected by AZA in unstimulated Ju.1 cells

<table>
<thead>
<tr>
<th>Downregulated Genes</th>
<th>Function</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>FK506-binding protein 1A</td>
<td>Signal transduction</td>
<td>-2.1</td>
</tr>
<tr>
<td>Protein tyrosine phosphatase</td>
<td>Signal transduction</td>
<td>-2.5</td>
</tr>
<tr>
<td>Cathepsin H</td>
<td>Protein metabolism</td>
<td>-2.9</td>
</tr>
<tr>
<td>CD28 Isoform 1</td>
<td>Signal transduction</td>
<td>-2.6</td>
</tr>
<tr>
<td>Alpha 4-integrin</td>
<td>Cell adhesion</td>
<td>-2.5</td>
</tr>
<tr>
<td>Protein-tyrosine kinase(DRT)</td>
<td>Signal transduction</td>
<td>-2.1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Upregulated Genes</th>
<th>Function</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>G protein-coupled receptor 64</td>
<td>Signal transduction</td>
<td>2.0</td>
</tr>
<tr>
<td>Seryl-tRNA synthetase</td>
<td>Protein metabolism</td>
<td>2.5</td>
</tr>
<tr>
<td>Eukaryotic translation initiation factor 4E binding protein(ETIF4B)</td>
<td>Protein metabolism</td>
<td>2.3</td>
</tr>
<tr>
<td>Phosphoserine phosphatase</td>
<td>Signal transduction</td>
<td>2.4</td>
</tr>
<tr>
<td>Protein kinase</td>
<td>Signal transduction</td>
<td>2.6</td>
</tr>
<tr>
<td>cAMP responsive element binding protein 1</td>
<td>Signal transduction</td>
<td>2.1</td>
</tr>
<tr>
<td>PMAIP1</td>
<td>Signal transduction</td>
<td>2.3</td>
</tr>
<tr>
<td>Phosphodiesterase 4C</td>
<td>Signal transduction</td>
<td>3.1</td>
</tr>
<tr>
<td>GABA-A receptor associated protein</td>
<td>Neurotransmitter</td>
<td>3.6</td>
</tr>
<tr>
<td>Phosphoglycerate dehydrogenase</td>
<td>Glucose metabolism</td>
<td>2.9</td>
</tr>
<tr>
<td>Methionine aminopeptidase</td>
<td>Protein metabolism</td>
<td>2.7</td>
</tr>
<tr>
<td>Chloride intracellular channel 4 (CLIC4)</td>
<td>Ion channel</td>
<td>2.5</td>
</tr>
<tr>
<td>Methylene tetrahydrofolate dehydrogenase</td>
<td>Tetrahydrofolate metabolism</td>
<td>2.8</td>
</tr>
<tr>
<td>Phosphoserine aminotransferase</td>
<td>Signal transduction</td>
<td>2.8</td>
</tr>
<tr>
<td>Ribosomal protein L18a</td>
<td>Protein metabolism</td>
<td>2.0</td>
</tr>
<tr>
<td>Protein</td>
<td>Function</td>
<td>Fold Change</td>
</tr>
<tr>
<td>-----------------------------------------------------</td>
<td>---------------------------------</td>
<td>-------------</td>
</tr>
<tr>
<td>Transcription factor 17</td>
<td>Transcription factor</td>
<td>2.2</td>
</tr>
<tr>
<td>VLDL receptor</td>
<td>Lipid metabolism</td>
<td>2.8</td>
</tr>
<tr>
<td>Heat Shock Protein, neuronal DNAJ-like 1</td>
<td>Stress response</td>
<td>3.3</td>
</tr>
<tr>
<td>Asparagine synthetase</td>
<td>Protein metabolism</td>
<td>4.1</td>
</tr>
<tr>
<td>Cystatin A</td>
<td>Cysteine-proteinase inhibitor</td>
<td>5.0</td>
</tr>
</tbody>
</table>
**Table 2.** Genes significantly affected by AZA in stimulated Ju.1 cells

<table>
<thead>
<tr>
<th>Downregulated Genes</th>
<th>Function</th>
<th>Fold change (Effect of stimulation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calreticulin</td>
<td>Calcium metabolism</td>
<td>-2.1 (-0.2)</td>
</tr>
<tr>
<td>Apo-2 ligand</td>
<td>Immune regulation</td>
<td>-2.0 (3.1)</td>
</tr>
<tr>
<td>TNF (ligand) superfamily, member 10</td>
<td>Immune regulation</td>
<td>-2.8 (4.0)</td>
</tr>
<tr>
<td>CD1C antigen</td>
<td>Immune function</td>
<td>-2.2 (0.9)</td>
</tr>
<tr>
<td>TNF receptor superfamily, member 7</td>
<td>Immune regulation</td>
<td>-2.8 (3.3)</td>
</tr>
<tr>
<td>Ras-GTPase activating protein</td>
<td>Signal transduction</td>
<td>-3.1 (0.4)</td>
</tr>
<tr>
<td>Zinc finger protein (purine binding TF)</td>
<td>Transcription factor</td>
<td>-2.3 (-0.6)</td>
</tr>
<tr>
<td>ATP:citrate lyase</td>
<td>Energy metabolism</td>
<td>-3.2 (-0.2)</td>
</tr>
<tr>
<td>KIAA0611</td>
<td>Unknown</td>
<td>-4.3 (3.2)</td>
</tr>
<tr>
<td>PDGF-A</td>
<td>Growth factor</td>
<td>-4.2 (2.2)</td>
</tr>
<tr>
<td>Novel IL 21 receptor</td>
<td>Cytokine</td>
<td>-2.1 (4.5)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Upregulated Genes</th>
<th>Function</th>
<th>Fold change (Effect of stimulation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alpha hydroxysteroid dehydrogenase</td>
<td>Steroid metabolism</td>
<td>2.8 (-1.1)</td>
</tr>
<tr>
<td>MNPEP</td>
<td>Protein metabolism</td>
<td>2.1 (-0.4)</td>
</tr>
<tr>
<td>Zinc finger protein 161</td>
<td>Transcription factor</td>
<td>2.6 (-0.6)</td>
</tr>
<tr>
<td>Guanylate binding protein 1</td>
<td>Tumor cell proliferation</td>
<td>2.7 (0.0)</td>
</tr>
<tr>
<td>G-protein signaling 2</td>
<td>Signal transduction</td>
<td>2.7 (-0.1)</td>
</tr>
<tr>
<td>Asparagine synthetase</td>
<td>Protein metabolism</td>
<td>2.5 (1.4)</td>
</tr>
<tr>
<td>Plasminogen activator</td>
<td>Serine protease</td>
<td>2.5 (-2.5)</td>
</tr>
<tr>
<td>Arginosuccinate synthetase</td>
<td>Protein metabolism</td>
<td>2.8 (-0.4)</td>
</tr>
<tr>
<td>Solute carrier family 22</td>
<td>Ion transport</td>
<td>4.0 (0.3)</td>
</tr>
<tr>
<td>Surfactant protein A binding protein</td>
<td>Unknown</td>
<td>3.9 (-2.8)</td>
</tr>
<tr>
<td>hCREM type 2</td>
<td>Signal transduction</td>
<td>2.7 (1.4)</td>
</tr>
<tr>
<td>Protein</td>
<td>Function</td>
<td>Score (Δ)</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>---------------------------</td>
<td>-----------</td>
</tr>
<tr>
<td>Oxidized LDL receptor</td>
<td>Lipid metabolism</td>
<td>2.7 (-1.3)</td>
</tr>
<tr>
<td>Zinc finger protein 42</td>
<td>Transcription factor</td>
<td>2.5 (-0.4)</td>
</tr>
<tr>
<td>Protein phosphatase 1B2</td>
<td>Signal transduction</td>
<td>2.5 (0.0)</td>
</tr>
<tr>
<td>HPF1 Protein</td>
<td>Transcription factor</td>
<td>3.2 (-0.8)</td>
</tr>
</tbody>
</table>
Figure 1

6-TGN, pmol/8x10^8 cells

Whole blood  Lymphocytes
Figure 3

A

![Graph showing relative mRNA levels for TRAIL, TNFRSF7, and α4-integrin with treatments]

- AZA
- Stimulation
- AZA + stimulation

B

![Graph showing relative mRNA levels for TRAIL, TNFRSF7, and α4-integrin with different concentrations]

- 1 µmol/l
- 3 µmol/l
- 10 µmol/l
Figure 4

A

TRAIL, ng/mg protein

0 10 20 30

Control AZA

Unstimulated Stimulated

B

TRAIL, ng/mg protein

0 5 10 15 20 25

0 1 24 48

ND

AZA, hours

C

TRAIL, ng/mg protein

0 5 10 15 20

0 0.01 0.1 1 10

AZA μmol/l

D

TRAIL, ng/mg protein

0 10 20 30 40

ND ND

Unstimulated Stimulated

AZA

Control AZA
Figure 5

A

TRAIL, ng/mg protein

Unstimulated  OKT3  OKT3 + IL-1  OKT3 + anti-CD28

Control  AZA

B

IL-2, pg/mg protein

Unstimulated  OKT3  OKT3 + IL-1  OKT3 + anti-CD28

Control  AZA
Figure 7

AZA \[\rightarrow\] 6-MP \[\rightarrow\] 6-TGN

TPMT

HPRT

XO

6-Thiouric acid

6-TG

T lymphocyte activation signal

Purine nucleotide

TRAIL

TNFRSF7

\(\alpha 4\)-integrin

Etc.

Immunity

Inflammation