Thiopurines inhibit bovine viral diarrhea virus production in a thiopurine methyltransferase-dependent manner

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The family Flaviviridae comprises positive-strand RNA viral pathogens of humans and livestock with few treatment options. We have previously shown that azathioprine (AZA) has in vitro activity against bovine viral diarrhea virus (BVDV). While the mechanism of inhibition is unknown, AZA and related thiopurine nucleoside analogues have been used as immunosuppressants for decades and both AZA metabolites and cellular genes involved in AZA metabolism have been extensively characterized. Here, we show that only certain riboside metabolites have antiviral activity and identify the most potent known antiviral AZA metabolite as 6-methylmercaptopurine riboside (6MMPr). The antiviral activity of 6MMPr is antagonized by adenosine, and is specific to BVDV and not to the related yellow fever virus. An essential step in the conversion of AZA to 6MMPr is the addition of a methyl group onto the sulfur atom attached to position six of the purine ring. Intracellularly, the methyl group is added by thiopurine methyltransferase (TPMT), an S-adenosyl methionine-dependent methyltransferase. Either chemically bypassing or inhibiting TPMT modulates antiviral activity of AZA metabolites. TPMT exists in several variants with varying levels of activity and since 6MMPr is a potent antiviral, the antiviral activity of AZA may be modulated by host genetics.

INTRODUCTION

Flaviviridae positive-strand RNA viruses are widespread veterinary and human pathogens divided into three genera: Flavivirus, Hepacivirus and Pestivirus. More than two billion people live in areas where members of the genus Flavivirus, including dengue and yellow fever virus (YFV), are endemic (Mackenzie et al., 2004). Hepatitis C virus (HCV), the only member of the genus Hepacivirus, infects more than 170 million people and its effects are the leading cause of liver transplantation (Lavanchy et al., 1999). The genus Pestivirus is made up of animal pathogens including bovine viral diarrhea virus (BVDV), which leads to production losses of $10–57 million per million calvings worldwide (Houe, 1999). A shared characteristic of all members of the family Flaviviridae is the absence of widely available, effective treatments. While interferon and ribavirin are the current standard treatment for HCV, many patients are not treated due to inadequate potency, expense and toxicity (Pawlotsky, 2003). The development of new antiviral drugs has been hampered by the lack of small-animal models. Despite considerable investment in developing new therapies for HCV, no new broad-spectrum antiviral drugs applicable to a range of flaviviruses have yet emerged.

Previous work in our laboratory has shown that azathioprine (AZA), a thiopurine immunosuppressant used in HCV-positive liver transplant patients, has in vitro activity against both BVDV and an HCV replicon (Stangl et al., 2004). These findings are supported by retrospective clinical trials, which show that AZA slows down the development of HCV-induced fibrosis in newly infected grafted livers after transplantation (Bahr et al., 2005; Hunt et al., 2001; Samonakis et al., 2005). As shown in Fig. 1, AZA is a nucleoside analogue prodrug that is cleaved to become the nitrogenous base, 6-mercaptopurine (6MP) and has anti-HCV replicon and anti-BVDV activity (Stangl et al., 2004). 6MP is ribosylated in cells to the 6MP-riboside, 6-thioinosine (6TI). 6TI is a branch point in thiopurine metabolism and can be either methylated to become 6-methylmercaptopurine riboside (6MMPr) or processed to 6-thioguanosine (6TGr). Each of these metabolites is known to have specific effects on cells, such as the inhibition of purine synthesis by 6MMPr or incorporation into cellular DNA of 6TGr that may be involved in antiviral effects (Coulthard et al., 2002; Ling et al., 1992; Vogt et al., 1993). Thiopurine metabolism is well characterized. Thiopurine methyltransferase (TPMT) is an S-adenosyl methionine-dependent methyltransferase that converts 6TI to 6MMPr.
Several genetic polymorphisms of TPMT, with a range of activities, exist in humans. Most people (90%) have moderate to high levels of TPMT activity leading to the rapid conversion of 6T to 6MMP. A smaller percentage (~10%) have intermediate activity and ~0.1% of people show little or no detectable TPMT activity (Otterness et al., 1997; Tai et al., 1997; Wang et al., 2003; Wang & Weinshilboum, 2006). Variations in activity are due to specific mutations in TPMT that increase its degradation and alter the dose of thiopurine required to achieve the desired efficacy. When AZA is used to treat autoimmune hepatitis or post-transplantation graft rejection, patients with very low TPMT activity require lower doses of AZA compared with patients with typical TPMT activity (Gisbert et al., 2006). In fact, Dervieux et al. (2002) measured intracellular concentrations of 6MMP and 6TGr in patients receiving AZA or 6MP and correlated nucleoside levels of 6MMP and 6TGr to patient TPMT activity. Patient TPMT activities can be predicted by sequencing or measured biochemically, making this one of the first clinical examples of a treatment tailored to patient genetics.

The goal of this research was to determine which metabolite of AZA has the most potent antiviral activity and to gain a better understanding of how this compound may act in vitro. Stangl et al. (2004) found that AZA, a nitrogenous base, exerted an antiviral effect on BVDV in vitro but, since high concentrations were required in vitro, whether this effect was clinically relevant was unclear. Our findings indicate that by delivering 6MMP as a riboside, the IC50 value for 6MMP is lowered to 1 μM, a concentration that is easily reached in patients receiving the prodrugs AZA or 6MP (Dervieux et al., 2005). We also show that upstream metabolites of 6MMP must be converted to 6MMP by TPMT to have full antiviral activity. Furthermore, cellular purine pools play a role in the antiviral effect. 6MMP is 100-fold more potent against HCV than thiopurines used previously and acts at concentrations that can be reached and tailored to patients based upon patient genetics.

**METHODS**

**Cell lines and viral strains.** All BVDV cell culture experiments were performed in Madin–Darby bovine kidney cells (MDBK) (Madin & Darby, 1958). MDBK cells were maintained in Dulbecco’s modified Eagle’s medium (Cellgro; Mediatech) supplemented with 10% fetal bovine serum (FBS) that was free of BVDV and BVDV antibodies (Atlanta Biologicals), 4.5 g glucose l⁻¹, 2 mM l-glutamine, 100 IU penicillin ml⁻¹, 100 IU streptomycin ml⁻¹, and 0.25 mg Amphotericin B ml⁻¹ at 37 °C and 5.0% CO2. Approximately 4 × 10⁵ MDBK cells were added to each well of a six-well plate and incubated at 37 °C and 5.0% CO2 for 4 h. Aliquots (200 μl) of serial 10-fold dilutions of supernatant were added and incubated for 1 h at 37 °C and 5.0% CO2. Supernatants were then removed and 2 ml 1.0% agarose + 1× growth medium overlay were added to each well. Plates were incubated for between 72 and 96 h until plaques were visible through the overlay, at which point the overlay was removed and cells were stained with 0.8% crystal violet (w/v) dissolved in 20% ethanol. Triplicate titrations were performed. Error bars represent sd.

**BVDV plaque assay.** Approximately 4 × 10⁵ MDBK cells were added to each well of a six-well plate and incubated at 37 °C and 5.0% CO2 for 4 h. Aliquots (200 μl) of serial 10-fold dilutions of supernatant were added and incubated for 1 h at 37 °C and 5.0% CO2. Supernatants were then removed and 2 ml 1.0% agarose + 1× growth medium overlay were added to each well. Plates were incubated for between 72 and 96 h until plaques were visible through the overlay, at which point the overlay was removed and cells were stained with 0.8% crystal violet (w/v) dissolved in 20% ethanol. Triplicate titrations were performed. Error bars represent sd.

**Cellular growth measurements.** Toxicity of thiopurines to MDBK cells was measured using the Cyquant cell proliferation kit from Invitrogen. Approximately 6.25 × 10⁴ MDBK cells were added to black 96-well plates (Corning) and incubated for 30 min. Thiopurines were added at concentrations of 10 μM and incubated for 4, 24 or 48 h. Cell proliferation was measured using the Cyquant kit according to the manufacturer’s instructions and fluorescence was measured on a Molecular Devices Spectramax Gemini EM fluorescence spectrophotometer. Cell growth experiments were performed similarly for Huh7 cells except that 2.5 × 10⁵ cells were plated and incubated for 24, 48 or 72 h before being read.

**Cellular toxicity measurements.** Cellular toxicity of thiopurines on MDBK and Huh7 cells was measured directly by lactate dehydrogenase release from cells by the CytoTox-ONE Homogeneous Membrane Integrity Assay from Promega. For MDBK cells, 6.25 × 10⁵ cells were incubated in black 96-well plates for 48 h with increasing concentrations of thiopurine nucleosides that were added 30 min after plating. Toxicity measurements were then taken according to the manufacturer’s instructions and a maximum value for cytotoxicity, based on detergent lysed cells, was used to calculate the percentage cytotoxicity. All treatments were performed in triplicate and read in triplicate on the fluorescence spectrophotometer described above. Huh7 cell toxicity measurements were done similarly except that 2.5 × 10⁵ cells were seeded into wells and incubated for 72 h.

**Secreted alkaline phosphatase (SEAP)/HCV replicon activity assays.** Stably transduced Ntat2Neo EN5-3 cells, derived from Huh7 human hepatoma cells, as described previously (Ikeda et al., 2002; Yi et al., 2002), were a kind gift from Stanley Lemon (University of Texas, Medical Branch, Galveston, TX, USA). The HCV replicon contains a single copy of the human immunodeficiency virus TAT protein 5’ to the HCV non-structural proteins. As the replicon RNA replicates, the amount of TAT protein generated corresponds to the amount of HCV protein translated. TAT activates transcription of (CEP), supernatants were removed and passed twice in MDBK cells to obtain a working stock of BVDV.

**Reagents.** 6-Mercaptopurine (6MP), 6-thioinosine (6TI), 6-thio-guanosine (6TGr), 6-methylmercaptopurine riboside (6MMPr), sulfasalazine, adenine, adenosine, inosine and guanosine were all purchased from Sigma-Aldrich. DMSO was from Fisher Scientific. All drugs were dissolved in sterile deionized warm water except for 6MP, 6TGr and sulfasalazine, which were dissolved in DMSO.
SEAP from a second plasmid contained in the cells. Thus, the amount of HCV replication can be measured by using SEAP in the cell culture supernatants, allowing the measurement of HCV replication in the same cells over time. In these cells, levels of SEAP directly correlate with HCV replicon RNA levels. Approximately 2 × 10⁴ cells were plated into each well of a 24-well plate. Cells were incubated overnight in the presence of 2 μg blasticidin (Invitrogen) ml⁻¹ and 1.0 mg G418 (Invitrogen) ml⁻¹. Blasticidin maintains the tat-transactivatable ENS-3 plasmid and G418 selects for the HCV replicon. Supernatants were removed and adherent cells were washed three times with PBS and 1 ml DMEM with 10 % FBS, 100 IU penicillin ml⁻¹, 100 μg streptomycin ml⁻¹ and blasticidin was added to each well. Drugs were added and cells were incubated for 2 days at 37 °C and 5.0 % CO₂. After 48 h, the supernatants were removed and the cells were washed twice with PBS. A 1 ml volume of DMEM with 10 % FBS, 100 IU penicillin ml⁻¹, 100 μg streptomycin ml⁻¹ and blasticidin was added to each well and incubated for 24 h. Supernatants were removed and SEAP activity was measured with the Phospha-Light assay system (Applied Biosystems).

YFV replicon synthesis and transfection. Plasmid YF-R.luc2A-RP, the cDNA for a YFV replicon containing Renilla luciferase in the same cistron as the non-structural proteins of YFV, was graciously provided by Richard Kuhn at Purdue University, USA (Jones et al., 2005). DNA was linearized by overnight digestion with Xhol and was ethanol precipitated. RNA was made with a mMessage mMachine from Ambion according to the manufacturer’s directions and cleaned up by LiCl precipitation. RNA was visualized on a gel to confirm size and transfected into HuH7 cells using the TransIT-mRNA transfection kit from Mirus. Approximately 1 × 10⁴ HuH7 cells were added to each well of a 12-well plate and incubated overnight at 37 °C and 5.0 % CO₂. A transfection mixture was made consisting of 100 μl Opti-Mem (Invitrogen), 1 μg RNA, 1 μl Boost (Mirus) and 2 μl TransIT-mRNA transfection reagent per well and incubated for 3 min at room temperature. After incubation, 100 μl transfection mixture was added to each well and the culture was incubated at 37 °C and 5.0 % CO₂ for 2 h before adding increasing concentrations of 6MMPr. Transfected cells were incubated for 36 h, at which time supernatants were removed and cells were washed with PBS. Cells were then lysed using Renilla lysis buffer provided in the Renilla Luciferase assay kit from Promega. Samples were read in triplicate with the Renilla Luciferase assay kit according to the manufacturer’s instructions on a Turner Designs 20/20n luminometer.

Statistical and data analysis. Student’s t-test was used to calculate all P-values. Graphs were plotted using Microsoft Excel X for Macintosh. Error bars represent s0 and IC₉₀ values were determined based on data in Fig. 3(a) as the drug concentration at which mock treatment viral titres were reduced by 90 %.

RESULTS

Ribose addition increases the antiviral effects of methylated thiopurines. Previously our laboratory found that AZA and its metabolite 6MP, a nitrogenous base, are antiviral for BVDV (Stangl et al., 2004). The addition of ribose to 6MP to make 6TI (Fig. 1) was hypothesized to lower the IC₉₀ since 6TI is one enzymic step downstream of 6MP. BVDV-infected MDBK cells were maintained with increasing concentrations of either 6TI or 6MP and after 48 h BVDV levels in supernatants were titrated. 6TI and 6MP show similar antiviral activity though 6TI is significantly, albeit slightly, more potent than 6MP at 5 μM (P=0.002, Fig. 2a). A similar experiment was performed for 6MMP and 6MMPr (Fig. 2b) and showed that, while 6MMP delivered as a base had little activity, the addition of a ribose to make 6MMPr increased BVDV inhibition more than 500-fold. The increase in antiviral effect between 6MMP and 6MMPr is quite striking and unexpected, since 6MMP and 6MMPr are traditionally thought of as by-products of thiopurine metabolism with little clinical importance.

6MMPr is the most potent anti-BVDV thiopurine

Since delivery of 6MMP as a ribose changed the antiviral activity so dramatically, we measured the antiviral effects of ribose addition to other thiopurine bases. A direct comparison of antiviral effects was performed between 6TI, 6MMPr and 6TGr to determine the most potent antiviral nucleoside metabolite of AZA. Thiopurines are known to have specific effects such as incorporation into DNA or decreasing de novo purine synthesis. Because of this, identifying the most potent antiviral was an important first step in narrowing the mechanism of inhibition. BVDV was incubated with increasing concentrations of each compound for 48 h and virus present in supernatants was titrated (Fig. 3a). 6MMPr was the most potent antiviral with an IC₉₀ of 1.0 μM causing nearly a 100-fold decrease in viral titres, while 6TGr did not reach IC₉₀ even at concentrations as high as 50 μM (data not shown). 6TI had an IC₉₀ value of 2.9 μM, which is threefold higher than the IC₉₀ of 6MMPr.

Thiopurines are cytostatic, not cytotoxic to MDBK cells

Thiopurines are known inhibitors of de novo purine synthesis, and are typically cytostatic (Koontz & Wicks, 1977). We sought to differentiate whether the antiviral activity was linked to cell death, inhibition of cell growth or more specific alterations in cell metabolism. Inhibition of cell growth was measured with the Cyquant cell proliferation assay kit, which measures cell proliferation via a dye that fluoresces when bound to nucleic acid. Cells were incubated at 10 μM, the highest concentration of thiopurine used, for 4, 24 or 48 h. Fluorescence was measured at 520 nm after excitation at 485 nm. All thiopurines showed similar inhibition of cell growth and no statistically significant variations in cytostatic effects were observed among any of the treated cells (Fig. 3b). 6TGr exhibited similar cytostatic effects to 6MMPr, yet had little or no antiviral activity at 10 μM (Fig. 3a) or even at very high doses (data not shown). To directly measure cytotoxicity, the Cytotox-ONE kit from Promega was used. In this assay, lactate dehydrogenase released from damaged or dead cells into the culture supernatant is measured by changes in nucleosides up to 500 μM for 48 h. After incubation, changes in fluorescence were measured. A cytotoxicity of 15 % was observed in untreated cells and all thiopurines showed cytotoxicity at or near this value (Fig. 3c). An MTS
kit (CellTiter 96 AQ
cous One Solution Proliferation Assay; Promega), as expected, showed a blockage of ATP synthesis in MDBK cells (data not shown; Berridge & Tan, 1993). Since thiopurines inhibit cellular proliferation, but only 6TI and 6MMPr and not 6TGr have an antiviral effect and no compound significantly increases cytotoxicity, the antiviral effect is more likely to be due to a more specific alteration of cellular metabolism than because of cytotoxicity.

TPMT activity is required for full antiviral effects of 6TI

TPMT is required for the conversion of 6TI to 6MMPr. To test whether 6TI must be converted to 6MMPr in order to have an antiviral effect, TPMT activity was inhibited in cells using sulfasalazine, a potent TPMT inhibitor. Sulfasalazine is a clinically used prodrug of 5-aminosalicylic acid that has been shown to block TPMT in vitro (Szmulanski & Weinshilboum, 1995; Woodson et al., 1983). During a 48 h infection, 6TI and 6MMPr concentrations were kept constant at 1 μM over a range of sulfasalazine concentrations. Viral titres were compared, sulfasalazine was found to decrease the antiviral effect of 6TI in a dose-dependent manner yet showed little effect on 6MMPr activity (Fig. 4). Similar results were observed when 5-aminosalicylic acid was used but the effects were not as pronounced (data not shown).

Adenosine, inosine and adenine decrease thiopurine anti-BVDV effects

6MMPr is a potent inhibitor of de novo purine synthesis and cellular purine pools may play a role in antiviral effects since exogenous addition of nucleosides to cells can block the antiviral activity of nucleoside analogues (Elion, 1989; Vogt et al., 1993). In order to measure the effects of purine pools on viral replication, guanosine (Fig. 5a), inosine (Fig. 5b) or adenine (Fig. 5c) was added during BVDV infection. MDBK cells were infected with BVDV at an m.o.i. of 0.001 and incubated with no drug, 10 μM 6TI or 6MMPr in the presence of 0–100 μM exogenous purines. Infections proceeded for 48 h and supernatants were
titrated by plaque assay. Guanosine alone had little effect on viral replication and did not relieve the antiviral effects of 6TI or 6MMPr (Fig. 5a). In fact, guanosine decreased viral titres in the presence of 6MMPr to below the limit of detection of the plaque assays used, which is 15 p.f.u. ml⁻¹.

Increasing concentrations of inosine, however, relieved the 80-fold inhibition of BVDV by both 6TI and 6MMPr (Fig. 5b). 6MMPr inhibition was decreased by nearly 10-fold, while 6TI effects were reduced by 25-fold in the presence of 100 μM inosine. Finally, the experiment was repeated using adenosine. Adenosine lessened 6MMPr inhibition by fivefold and lessened 6TI inhibition by 40-fold (Fig. 5c). Since 6TI and 6MMPr uptake is dependent upon equilibrative nucleoside transporter 1 (ENT1), and since ENT1 can be inhibited by millimolar concentrations of adenosine, it is possible that the decreases in antiviral effects are due to a decrease in cellular uptake of

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**Fig. 2.** Effects of ribose addition on thiopurine antiviral activity. (a) MDBK cells infected with BVDV at an m.o.i. of 0.001 were treated with increasing concentrations of 6MP (○) or its ribosylated derivative 6TI (△) for 48 h at 37 °C and 5.0 % CO₂ and supernatants were harvested and titrated. (b) As in (a), BVDV-infected cells were treated with increasing concentrations of 6MMP (□) or ribosylated 6MMPr (○) for 48 h before titration. Values have been normalized to mock-treated cells and error bars represent sd. Student’s t-test was used to calculate P-values.

**Fig. 3.** Effects of thiopurine nucleosides on viral and cellular replication. (a) MDBK cells were infected with BVDV at an m.o.i. of 0.001 and incubated in the presence of thiopurine nucleosides for 48 h. Nucleosides used were 6TGr (□), 6TI (△) and 6MMPr (○). (b) The effects of thiopurine nucleosides on MDBK cell proliferation. Cells were split and incubated in 96-well plates for 48 h. Nucleic acids in cells were measured with the Cyquant kit (Invitrogen) to determine how thiopurines affect cellular growth. All nucleosides were used at a 10 μM final concentration. (c) Thiopurine toxicity measurements. MDBK cells were split into black 96-well plates and effects of thiopurine nucleosides on cellular toxicity were measured using the CytoTox-ONE assay system from Promega. Cells were incubated in the presence of increasing thiopurines for 48 h. Percentage cytotoxicity was measured by comparing treated cells with mock-treated cells that were lysed with detergent. Cytotoxic concentration (CC₅₀) values for all compounds tested in MDBK cells were greater than 500 μM, as measured by lactate dehydrogenase release.
thiopurines via direct competition (Fotoohi et al., 2006a, b). To rule this out as the primary effect, the above add- 
back experiment was repeated with the addition of adenine, 
which has been shown previously to have little effect on 
thiopurine uptake (Fotoohi et al., 2006b). Adenine 
(200 μM) blocked 6TI antiviral activity by greater than 
15-fold and 6MMPr by 5.5-fold (Fig. 5d), suggesting that 
uptake inhibition is not the principal mechanism by which adenosine inhibits 6MMPr activity.

6MMPr but not 6TI inhibits an HCV replicon in Huh7 cells

Ntat2ANeo cells containing an HCV replicon were treated 
with increasing concentrations of 6TI and 6MMPr (0– 
60 μM) to measure the effects of thiopurines on HCV 
replication. Samples were read after 24 h of SEAP 
accumulation following treatment with 10 μM 6TI or 
6MMPr for 48 h. 6TI was found to have little anti-HCV 
activity, whereas 6MMPr inhibited SEAP production 
almost 10-fold (Fig. 6a).

Thiopurines slow Huh7 growth

Using the Cyquant assay described above, Huh7 cells were 
treated with 10 μM 6TI or 6MMPr or left untreated for 24, 
48 or 72 h (Fig. 6b). 6MMPr slowed cellular proliferation, 
yet did not stop growth. Again, direct effects of thiopurines 
on cellular toxicity were measured in Huh7 cells using the 
CytoTox-ONE assay as described for MDBK cells above. 
After 72 h, the amount of cytotoxicity observed for 6TI or 
6MMPr was not above the 8% cytotoxicity seen in untreated 
cells (Fig. 6c). These data, along with the anti-HCV effects 
seen in Fig. 6(a) suggest that the inhibition of HCV is not 
due to cellular toxicity.

6MMPr has little effect on YFV replication in Huh7 cells

YFV is the type member of the family Flaviviridae, genus 
Flavivirus. Cytostatic effects and virus-specific effects of 
6MMPr were tested using a YFV replicon in Huh7 cells. 
YF-R.luc2A-RP is a YFV replicon that contains Renilla 
luciferase in the same cistron as the non-structural proteins 
(NS1–NS5) of YFV. After transfection, cells were incubated 
for 2 h before addition of increasing concentrations of 
6MMPr. Cells were incubated for 36 h after 6MMPr 
addition and lysed, and luciferase activity was measured. As 
shown in Fig. 6(d), 6MMPr had little effect on YFV 
replication since the replicon was unaffected by the 
presence of 6MMPr. Other data showed that a replication- 
deficient YFV replicon was unaffected by 6MMPr, 
suggesting that translation is not the target of 6MMPr antiviral activity (data not shown). This experiment was 
performed in the same cells as the HCV replicon experiments and there was little antiviral activity towards 
YFV. This further strengthens the hypothesis that 6MMPr antiviral effects are independent of cytostatic effects 
because a similar virus is able to grow in Huh7 cells 
treated with 6MMPr under conditions in which HCV 
replication is inhibited. This indicates that a selective effect 
of 6MMPr is responsible for the antiviral effects.

DISCUSSION

Few drugs are effective against members of the family 
Flaviviridae. Since these viruses replicate their RNA with a 
high error rate, it is important to develop multiple drugs that 
inhibit different steps in the viral life cycle to slow the 
development of resistance. Here, we report that the addition 
of a ribose to thiopurine nitrogenous bases increases activity 
and lowers IC₉₀ values to pharmacologically achievable 
fractions for patients on AZA or 6MP (Dervieux et al., 
2002, 2005). The increase in antiviral activity between 
6MMPr and 6MMP was particularly surprising since 
6MMP, the base alone, had no antiviral effects (Fig. 2b). 
While recently thiopurine use has been supplanted by more 
potent immunosuppressants, thiopurines are a well-studied 
group of compounds that are not only safe and effective, but 
widely used, with an extensive historical record over more 
than four decades (Elion, 1989).

6MMPr may antagonize several viral and cellular processes, 
yet we put forward three separate arguments that indicate 
that the antiviral effect of 6MMPr is not a non-specific 
cellular effect. Firstly, 6MMPr and 6TGr, which show 
similar cytostatic effects, have markedly different effects on 
viral replication. Fig. 3(a) shows that 6MMPr inhibits viral 
replication almost 100-fold, while 6TGr has little if any 
effect on BVDV replication. Secondly, by comparing the 
effects of 6MMPr on related replicons in the same Huh7
cell line (Fig. 6a, d), we show that the anti-replication effects of 6MMPr are virus-, not cell type-specific, since the HCV replicon is strongly inhibited, while there is no effect on YFV replication. Finally, Stangl et al. (2004) found that 6MP still had antiviral effects in the presence of thymidine, a cytostatic cell cycle blocker, suggesting that the antiviral effects are independent of cell cycle blockage and cytostatic effects.

There are many potential mechanisms for thiopurine inhibition of BVDV and HCV. Since the HCV replicon contains only the machinery for HCV replication and since 6MMPr inhibits the HCV replicon, antiviral effects are most likely to be due to effects on RNA replication and not effects on viral entry, packaging or release. The mechanisms by which nucleotide analogues inhibit other RNA viruses include incorporation of analogues into RNA, increasing viral mutagenesis or acting as chain terminators of viral RNA (D’Abramo et al., 2004; Graci & Cameron, 2002; Koh et al., 2005). These mechanisms require phosphorylation and studies have shown that 6MMPr is phosphorylated in red blood cells (Dervieux et al., 2002; Stet et al., 1994). The data in Fig. 5(a) indicate that 6MMPr and guanosine act synergistically. While, surprisingly, conversion of guanosine to GTP requires the addition of three phosphate groups to guanosine and the addition of exogenous guanosine may have depleted ATP levels further in MDBK cells, potentiating 6MMPr anti-BVDV effects just as flooding cells with adenosine decreased the antiviral effects. Stollar & Malinoski (1981) found that exogenous

Fig. 5. Purine add-back effects on 6TI and 6MMPr BVDV inhibition. MDBK cells were infected with BVDV at an m.o.i. of 0.001 and mock treated (□) or incubated with 10 μM 6TI (○) or 10 μM 6MMPr (○) for 48 h. Increasing concentrations of various purines, guanosine (a), inosine (b), adenosine (c) and adenine (d), were added to each infection. Supernatants were removed and titrated. In (a), the combination of 10 μM 6MMPr and 100 μM guanosine decreased viral titres to below the level of detection of the plaque assay, 15 p.f.u. ml⁻¹, as indicated by the horizontal dashed line. Values shown were normalized to the untreated and mock-infected controls for each experiment.
guanosine decreased cellular ATP levels in a mosquito cell line, and a similar phenomenon could be occurring here. Data shown in Fig. 5 indicate that purine pools play a role in the antiviral activity, but this still leaves several possibilities open for potential mechanisms. Decreased purine pools lower the amount of nucleotides available for the production of viral RNA during infection and may increase the chance of misincorporation or chain termination.

Crystal structures of both the HCV and BVDV NS5B RNA-dependent RNA polymerases show allosteric nucleoside/nucleotide-binding sites that could alter the properties of NS5B (Bressanelli et al., 2002; Choi et al., 2004). 6MMPr may compete with other nucleosides for binding at these sites. Crystallography and biochemical data also indicate that BVDV and HCV undergo de novo initiation of viral RNA synthesis (Bressanelli et al., 2002; Butcher et al., 2001; Zhong et al., 2000). Viral RNA is copied in a primer-independent manner that initiates from a single GTP bound near the active site of NS5B. 6MMPr may interfere with GTP binding at the initiation site, thereby decreasing viral RNA replication initiation. The precise mechanism by which 6MMPr achieves this inhibition is not yet clear. 6MMPr may also compete with other nucleosides for binding at the active site and affect substrate binding.

**Fig. 6.** (a) Antiviral activity of thiopurines against HCV replicon activity. Huh7 cells containing the SEAP-producing HCV replicon were incubated with increasing concentrations of 6TI (△) or 6MMPr (○) for 2 days. HCV replicon activity was measured in these cells by SEAP activity in the supernatants. After 2 days, supernatants were removed and cells were washed three times with PBS and new medium containing the drug was added. Cells were then incubated for 24 h and supernatants were removed. SEAP production was measured by the Phospha-Light chemiluminescent kit from Applied Biosystems. (b) Effects of thiopurines on Huh7 cell growth. Huh7 cells were incubated with 6TI (□) or 6MMPr (○) at 10 μM or left untreated (○) for 24, 48 or 72 h. Cellular proliferation was measured using the Cyquant cellular proliferation kit and fluorescence correlates to the amount of nucleic acid present. (c) Cytotoxic effects of thiopurines on Huh7 cells. Huh7 cells were seeded into black 96-well plates and incubated with increasing concentrations of 6TI (△) or 6MMPr (○). Toxicity measurements were performed with the CytoTox-ONE assay kit from Promega. The CC50 values for all compounds tested were greater than 50 μM in Huh7 cells. (d) Huh7 cells transfected with a YFV replicon (○) that contains Renilla luciferase and exposed to increasing concentrations of 6MMPr 2 h after transfection. Supernatants were removed and cells were washed and then lysed 36 h after 6MMPr addition. The amounts of luciferase in cell lysates were read and compared.
which 6MMPr inhibits HCV and BVDV replication remains elusive and may be a combination of effects that lead to the potent inhibition of RNA virus replication.

In this report, two lines of evidence indicate that conversion of 6TI to 6MMPr is essential for thiopurine antiviral activity. Firstly, sulfasalazine, a benzylc acid inhibitor of TPMT, decreased 6TI inhibition of BVDV, showing that 6MMPr is responsible for at least a proportion of the antiviral effect of thiopurines (Fig. 4). Secondly, Fig. 6(a) and (b) suggest that 6TI is most likely not being converted to 6MMPr or is unable to penetrate Huh7-derived Nat2ANeo cells, since 6TI has no effect on the HCV replicon. TPMT mRNA is present in Huh7 cells, as detected by RT-PCR, and protein is expressed, as confirmed by Western blot (data not shown). Variation in TPMT activity has been detected in patients receiving AZA or 6MP and predicts 6MMPr nucleotide levels. Since some HCV-transplant patients receive AZA to prevent graft rejection, it is possible that variations in 6MMPr levels could affect the presence or absence of an antiviral effect (Dervieux et al., 2002; Otterness et al., 1997). Since 6MMPr is the most potent thiopurine antiviral, the immunosuppressant effects of AZA and 6MP can be separated from antiviral effects by giving 6MMPr directly.

Multivariate analysis of clinical data suggests that AZA treatment increases patient survival post-transplant and decreases liver fibrosis (Bahr et al., 2005; Hunt et al., 2001; Kornberg et al., 2005; Samonakis et al., 2005). Interestingly, the enzyme found to be necessary for the inhibition of BVDV by thiopurines, TPMT, exists as several different variants in the human population, leading to tailored treatments based on TPMT polymorphisms (Wang & Weinshilboum, 2006). Prospective studies of AZA-treated liver transplant patients in which thiopurine nucleotide levels are measured in liver biopsies and correlated with survival would be useful. Patients homozygous for the most common TPMT alleles reach 6MMPr erythrocyte concentrations of 30 μM on AZA therapy, while patients with heterozygous activity reach concentrations of 6 μM. If the protection from HCV-mediated fibrosis shown in clinical trials is due to the antiviral activity of thiopurines then we would anticipate that the effect is largest in those patients that accumulate the most 6MMPr intracellularly. 6MMPr levels in the liver after AZA therapy can be largely predicted by TPMT activity, therefore a retrospective study correlating TPMT polymorphisms to AZA treatments and HCV progression may provide further evidence of a protective effect by AZA or other thiopurines and lead to tailored immunosuppressive therapies for each HCV-infected liver transplant patient.

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