The effect of a novel transition state inhibitor of methylthioadenosine phosphorylase on pemetrexed activity

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Abstract

Pemetrexed is a new-generation antifolate inhibitor of thymidylate synthase (TS) and a weaker inhibitor of glycaminide ribonucleotide transformylase (GARFT) required for de novo purine synthesis. Methylthioadenosine phosphorylase (MTAP) salvages purines by releasing adenine from methylthioadenosine and is often deleted in mesothelioma. The current study addresses the effect of MTAP on pemetrexed activity using a highly potent transition state inhibitor of MTAP, MT-DADMe-Immucillin A (ImmA; Ki = 86 pmol/L) in the MTAP(+) NCI-H28 and MTAP(−) NCI-H2052 mesothelioma cell lines. Based on selective nucleoside protection, TS was found to be the primary pemetrexed target in both cell lines with GARFT inhibition requiring 20- to 30-fold higher pemetrexed concentrations. ImmA had no effect on pemetrexed activity but, when thymidine was added, the pemetrexed IC50 decreased by a factor of ~3 in MTAP(+) H28 cells with no effect in MTAP(−) H2052 cells. Conversely, the transfection of MTAP into H2052 cells increased the pemetrexed IC50 by nearly 3-fold but only in the presence of thymidine; this was reversed by ImmA. An MTAP-specific short interfering RNA produced a 2-fold decrease in pemetrexed IC50 in MTAP(+) HeLa cells in the presence of thymidine. These data indicate that suppression of constitutive MTAP has no effect on pemetrexed activity when the primary target is TS. There is a modest salutary effect when the pemetrexed target is GARFT alone. [Mol Cancer Ther 2006;5(10):2549–55]

Introduction

Pemetrexed (Alimta) is a new-generation antifolate recently approved, and in clinical use, for the treatment of mesothelioma and non–small cell lung cancer (1, 2). The antitumor activity of pemetrexed is primarily a consequence of inhibition of thymidylate synthase (TS). There is also weaker inhibition of glycaminide ribonucleotide transformylase (GARFT). In cell-free systems, the Ki of pemetrexed pentaglutamate for TS is 1/50th that of GARFT (3).

Methylthioadenosine phosphorylase (MTAP) is an enzyme that releases adenine and methionine from methylthioadenosine, a byproduct of the polyamine synthetic pathway. This purine salvage has the potential for antagonizing the effects of agents that inhibit purine synthesis. The MTAP gene is codeleted with p16 in the majority of mesothelioma tumor samples (4), and it has been suggested that the activity of pemetrexed in this disease may be related to the loss of MTAP (5). Previous studies suggest that MTAP deletion has a salutary effect on the activity of other antifolates such as methotrexate, trimetrexate, and lomefox, as well as the purine synthesis inhibitor L-alanosine (6–10). However, these studies compared nonsyngeneic cell lines or evaluated the effect of MTAP overexpression and, until this time, only a weak inhibitor of MTAP was available to assess the role of constitutive enzyme activity (7). Suppression of constitutive MTAP expression by interfering RNA (siRNA) was shown in one report to enhance L-alanosine activity (11). There have been no studies that have addressed the effect of MTAP expression on pemetrexed activity.

The current study employed several experimental approaches to assess the role of MTAP on pemetrexed activity in human mesothelioma and HeLa cell lines. First, a highly potent (Ki = 86 pmol/L) transition state inhibitor of MTAP, MT-DADMe-Immucillin A (ImmA) developed at this institution, was used to suppress endogenous MTAP activity (12). Second, these results were compared with studies in which MTAP was silenced by stable siRNA transfection. Finally, the effect of MTAP transfection was assessed in an MTAP(−) mesothelioma cell line.

Materials and Methods

Chemicals and Cell Lines

Pemetrexed was provided by Eli Lilly and Company (Indianapolis, IN). MT-DADMe-ImmA was synthesized as previously reported (12). HeLa and NCI-H28 cells were obtained from American Type Tissue Culture Collection (Manassas, VA). The human mesothelioma cell line, NCI-H2052, was obtained from the National Cancer Institute (Bethesda, MD).

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Cell Culture and Growth-Inhibition Studies

Cells were grown in folate-free RPMI medium containing 10% dialyzed fetal bovine serum, 100 units/ml of penicillin, 100 μg/ml of streptomycin, 2 mmol/L of glutamine, and 20 μmol/L of β-mercaptoethanol. The medium folate growth source was 25 mmol/L of 5-formyltetrahydrofolate. For growth-inhibition studies, cells were seeded in 96-well plates at a density of 2,000 cells/well (1,000 cells/well for HeLa clones) and exposed to 11 different concentrations of pemetrexed for 6 days; the 12th well was drug-free. Cell growth was then quantified using a sulforhodamine B reagent (13). To assess the relative inhibition of TS and GARFT, the end products of these reactions—thymidine (10 μmol/L), hypoxanthine (100 μmol/L), or both—were added to the medium. To determine the effect of MTAP inhibition, 10 μmol/L of ImnA, the highest nontoxic concentration of this drug, was included in the medium.

Northern Blot Analysis

Total RNA was isolated from H28, H2052, and HeLa cells using TRIzol reagent (Invitrogen, Carlsbad CA). Thirty micrograms of this RNA was run on a 1% formaldehyde denaturing gel and blotted onto a Nytran N-membrane (Schleicher & Schuell, Keene, NH). A cDNA probe containing the entire open reading frame of MTAP was used to determine MTAP expression.

Stable Transfection of MTAP

The MTAP gene was previously cloned into the bacterial vector, pQE32 (12). The MTAP insert was excised from this vector using EcoRI and BamHI restriction enzymes and cloned into the pcDNA3.1+ vector (Invitrogen) which contains the G418 resistance gene. The insert is a fragment identical to MTAP cDNA (GenBank accession no. NM_002451) from bases 89 to 1024, which includes the entire open reading frame of MTAP (bases 114–962). Cloning was confirmed by sequencing using standard pcDNA3.1 forward and reverse primers following the manufacturer’s protocol. The MTAP plasmid was transfected into MTAP-null H2052 cells using LipofectAMINE Plus reagent (Invitrogen), followed 48 hours later by G418-selective pressure (500 μg/mL). A clone (M11) over-expressing MTAP was screened by quantitative reverse transcription-PCR (RT-PCR) and picked for further studies. Empty pcDNA3.1+ vector was similarly stably transfected into H2052 cells and a randomly picked vector control clone (V2) was obtained. These H2052 clones were maintained under G418-selective pressure.

MTAP Suppression by siRNA

Because MTAP(+) H28 cells are slow-growing and difficult to work with, MTAP(+) HeLa cells were used to silence MTAP siRNA expression. Using the Silencer Express (human U6) kit (Ambion, Austin, TX), four targets in the MTAP open reading frame were picked and siRNA expression cassettes (SEC) were constructed for these targets. A negative control siRNA expression cassette was also constructed following the manufacturer’s protocol. These constructs were transiently transfected into HeLa cells using LipofectAMINE Plus and best constructs screened using quantitative RT-PCR. The construct that caused maximum silencing of MTAP expression generated a 21-nucleotide siRNA complimentary to MTAP starting at position 554 (NM_002451). The MTAP siRNA and negative siRNA expression cassettes were cloned into the pSEC hyg ro vector (which contains the gene for hygromycin resistance) and confirmed by sequencing. These plasmids were then transfected into HeLa cells and stable clones were obtained using hygromycin-selective pressure (350 μg/mL). Clones from the MTAP siRNA group were screened by quantitative RT-PCR and three clones (5-6, 5-10, and 5-A) were picked for the study. Three randomly selected negative siRNA clones (N-1, N-3, and N-A) were also picked for comparison. These HeLa clones were maintained under hygromycin-selective pressure.

Quantitative RT-PCR

To assess MTAP expression, RNA was first isolated from HeLa and H2052 clones using TRIzol reagent (Invitrogen). Complimentary DNA was then synthesized from 5 μg RNA starting template using Superscript II kit (Invitrogen). This cDNA was diluted 1:8 and used for quantitative real-time PCR using the ABI 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA) and Sybr Green for product quantitation. The MTAP primers used were reported previously (14) and named MTAP forw2 (5′-GGG AAC ATC TGG GCT TTG-3′) and MTAP rev2 (5′-GCA CCG GAG TCC TAG CTT C-3′). β-Actin was used to normalize MTAP expression using previously reported primers (15): sense primer, 5′-CGTGTCTGCTGACCGAGC-3′ and antisense primer, 5′-GAAGGTCTCAAACATGATC-3′. These primers, only a single band is detected when PCR products are run in a 2% agarose gel. The quantitative PCR reactions were set-up in triplicate in 384-well plates in 8 μL reactions containing 2 μL of 1:8 diluted cDNA template, 0.5 μmol/L of MTAP primers, and Sybr Green Master mix (Applied Biosystems). One cDNA sample, diluted serially from 1:4 to 1:64, was used to plot standard curves for MTAP and β-actin expression. The cycling parameters were initial denaturation at 95°C for 10 minutes followed by 40 cycles at 95°C for 10 seconds, at 60°C for 20 seconds, and at 72°C for 30 seconds. SDS 2.0 software (Applied Biosystems) was used to analyze results and the standard curve method was used to calculate differences in MTAP expression following the manufacturer’s protocol.

Western Blotting

Cells (1 × 10⁶) were suspended in 100 μL of suspension buffer (100 mmol/L NaCl, 10 mmol/L Tris, 1 mmol/L EDTA) containing protease inhibitor (Sigma, St. Louis, MO) and lysates were prepared by sonication followed by centrifugation. Supernatants were used for protein determination using a bicinchoninic acid reagent (Pierce, Rockford, IL). Fifteen micrograms of protein were used for HeLa cell samples, whereas 5 μg of protein were used for H2052 cell samples. The protein samples were mixed with 2× sample loading buffer containing DTT and denatured at 95°C for 10 minutes prior to loading. Protein was separated in a 12% SDS-PAGE gel then blotted onto a Hybond-P.
polyvinylidene fluoride membrane (Amersham, Piscataway, NJ). Following blocking with 5% milk solution in TBS-T, the membrane was probed with MTAP primary antibody at a dilution of 1:5,000 in TBS-T containing 1% milk at room temperature for 1 hour. The rabbit MTAP polyclonal antibody was a generous gift from Chandan Guha (Albert Einstein College of Medicine). Anti-rabbit secondary antibody (Cell Signaling, Danvers, MA) was used at a dilution of 1:10,000 in TBS-T at room temperature for 1 hour. The enhanced chemiluminescence plus detection system (Amersham) was used to detect the signal. The MTAP antibody was then stripped off the membrane by incubation with 62.5 mmol/L Tris-HCl, 100 mmol/L β-mercaptoethanol, 10% SDS for 30 minutes at 50°C, then blocked again with 5% milk, and reprobed with mouse β-actin antibody (Sigma) using identical conditions except that the secondary antibody used was anti-mouse (Cell Signaling).

Clonogenic Assay
H2052 cells stably transfected with MTAP (M11) or empty vector (V2) were each seeded in two six-well plates at a density of 175 cells/well in medium containing 10 μmol/L thymidine. ImmA was added to one plate of each clone to achieve a final concentration of 10 μmol/L. Pemetrexed was then added to five wells of each plate at 3-fold serial dilutions of from 72.9 to 0.9 μmol/L for M11 cells and from 24.3 to 0.3 μmol/L for V2 cells. The sixth well was left drug-free. Medium was replaced every 5 days with the same constituents. Clones visible after 15 days were stained with the sulforhodamine B reagent and counted.

Results
TS versus GARFT Inhibition in MTAP Positive and Negative Cells
Two mesothelioma cell lines were picked to study the role of MTAP on pemetrexed antipurine activity. One, H28, was reported to be MTAP-positive and the other, H2052, MTAP-negative (4); this was confirmed by Northern blot analysis (data not shown). To dissect the relative importance of TS and GARFT inhibition in these two cell lines, the end products of these enzymes, thymidine, hypoxanthine, or both were added to the medium containing 10 μmol/L thymidine. ImmA was added to one plate of each clone to achieve a final concentration of 10 μmol/L. Pemetrexed was then added to five wells of each plate at concentrations of from 72.9 to 0.9 μmol/L for M11 cells and from 24.3 to 0.3 μmol/L for V2 cells. The sixth well was left drug-free. Medium was replaced every 5 days with the same constituents. Clones visible after 15 days were stained with the sulforhodamine B reagent and counted.

Figure 1. Pemetrexed inhibition of TS versus GARFT in MTAP-positive H28 (top) or MTAP-negative H2052 cells (bottom). Cells were grown with folate-free RPMI containing 25 nmol/L of 5-formyltetrahydrofolate and 10% dialyzed serum. Pemetrexed growth inhibition was assessed in the presence of thymidine (Thd) alone (●), thymidine (Thd) alone (●), or both thymidine and hypoxanthine (▲). Points, mean average of three experiments; bars, ±SE.

Figure 2. Effect of a highly potent MTAP inhibitor on pemetrexed antipurine activity in MTAP positive H28 (top) or MTAP-null H2052 cells (bottom). Pemetrexed growth inhibition was assessed in the presence of thymidine (Thd) in cells grown in folate-free RPMI containing 25 nmol/L of 5-formyltetrahydrofolate and dialyzed serum in the presence (●) or absence (●) of 10 μmol/L of the MTAP inhibitor ImmA. Points, mean average of three experiments; bars, ±SE.

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hypoxygenine. MTAP(−) H2052 cells were somewhat more sensitive overall to pemetrexed (IC50 ~ 40 nmol/L; Fig. 1, bottom), but the IC50 was increased ~20-fold to ~1 μmol/L when thymidine was added. Again, full protection required the addition of both thymidine and hypoxygenine (Fig. 1, bottom). Hence, TS was the primary target in both cell lines and the difference between pemetrexed concentrations required to deplete cells of thymidine and purines was comparable in both cell lines whether or not MTAP was present.

**Effect of a Highly Potent MTAP Inhibitor on GARFT Inhibition by Pemetrexed**

To evaluate the role of MTAP-mediated purine salvage on the antipurine effect of pemetrexed in the same cell line, a highly potent (Ki = 86 pmol/L) transition state MTAP inhibitor, ImmA, was used. Alone, this inhibitor had no effect on cell growth in both cell lines up to a concentration of ~10 μmol/L (data not shown). However, when MTAP(+) H28 cells were exposed to 10 μmol/L of ImmA in the presence of thymidine, the pemetrexed IC50 was decreased ~3-fold to ~1 μmol/L (Fig. 2, top) but there was no significant effect of ImmA in the MTAP(−) H2052 cells (Fig. 2, bottom). ImmA had no effect on pemetrexed activity in the absence of thymidine in H28 cells (data not shown). Hence, the antiproliferative effect of pemetrexed can be enhanced by ImmA but only when the target is GARFT.

**Effect of MTAP Overexpression or Suppression on Pemetrexed Activity**

To further confirm that the augmentation of pemetrexed antipurine activity by ImmA was solely a result of inhibition of MTAP, the gene was stably transected into MTAP(−) H2052 cells. As depicted in Fig. 3, a clone was picked (M11) that expressed very high levels of MTAP as assessed by quantitative RT-PCR (Fig. 3A) and by Western blotting (Fig. 3B) as compared with the absence of expression in the wild-type cells. Pemetrexed activity was then assessed in these cells and compared with cells stably transfected with empty vector (V2). As shown at the top of Fig. 4, the pemetrexed IC50 in the presence of thymidine was increased by ~3-fold in MTAP-transfected cells (M11) as compared with vector control cells (V2). Despite the high degree of MTAP overexpression in M11 cells, resistance to pemetrexed in the presence of thymidine was completely reversed by the addition of ImmA. No differences were detected in the absence of thymidine, indicating that the effects of MTAP expression were restricted to pemetrexed antipurine activity (Fig. 4, bottom).

To control for clonal variability, untransfected wild-type H2052 cells were also studied and their IC50s were nearly identical to that of vector control V2 cells (data not shown). The ~3-fold resistance to pemetrexed in the presence of thymidine in M11 cells compared with V2 cells was confirmed by a clonogenic assay (data not shown). The resistance pattern in the clonogenic assay was also completely reversed with 10 μmol/L of ImmA.

Finally, the effect of MTAP suppression by siRNA in MTAP(+) cells was evaluated. Because H28 cells are slow-growing when seeded at low density and form clones poorly, MTAP(+) HeLa cells were used instead. HeLa cells were stably transfected with a plasmid

![Figure 3](image-url)
encoding an siRNA specific for MTAP or with a negative siRNA plasmid and three clones from each group were picked for study. As depicted in Fig. 3A, MTAP gene expression was found to be reduced by 50% to 75% in the MTAP siRNA-transfected clones as measured by quantitative RT-PCR. This was associated with marked suppression of MTAP protein expression (Fig. 3B). This reduction in MTAP expression led to a 2-fold decrease in the average pemetrexed IC\textsubscript{50} in the presence of thymidine (Fig. 5, top) as compared with the negative siRNA clones. ImmA, at a concentration of 10 \( \mu \)mol/L, which is nontoxic to HeLa cells (data not shown), resulted in a ~2-fold reduction in the average pemetrexed IC\textsubscript{50} in the negative siRNA clones, and a small further decrease in pemetrexed IC\textsubscript{50} in the MTAP siRNA clones suggesting the suppression of residual MTAP activity in the latter cells in the presence of thymidine. There was no effect of the MTAP siRNA or ImmA in the absence of thymidine (Fig. 5, bottom).

**Discussion**

This study was undertaken to explore the effect of MTAP-mediated purine salvage on pemetrexed activity. The approach was novel in that a recently developed highly potent transition state inhibitor of MTAP was used to abolish constitutive enzyme activity so that the actions of pemetrexed could be compared in the same cell line with and without MTAP-mediated purine salvage. The results confirm in a mesothelioma cell, what has been seen in a variety of other cell lines, that pemetrexed is primarily a TS inhibitor and that inhibition of GARFT requires drug concentrations substantially higher than the levels required to suppress TS (3, 16–18). Although clinical pharmacokinetics with pemetrexed show that the drug’s plasma concentrations are, over brief intervals, substantially higher than what is required to suppress GARFT, the contribution of GARFT inhibition to the antitumor activity of pemetrexed is not clear (19). The data indicate that in the absence of added nucleoside substrates that circumvent the suppression of pemetrexed enzyme targets, the inhibition of MTAP had no effect on pemetrexed activity. However, under conditions in which pemetrexed action was restricted to GARFT, as with the addition of thymidine, suppression of MTAP produced a ~3-fold enhancement in the activity of this agent. A small, comparable suppression of MTAP-mediated purine salvage was also shown in MTAP(+) HeLa cells by gene silencing with siRNA but, again, only in the presence of thymidine. Finally, transfection of MTAP into an MTAP(-)/C0 mesothelioma cell line that achieved a high level of protein expression produced a ~3-fold decrease in pemetrexed activity only in the presence of thymidine. Taken together,
the data indicate that when constitutive levels of MTAP are suppressed, there is a modest increase in the antipurine effect of pemetrexed and no effect when TS is the primary target.

Previous reports have evaluated the role of MTAP in the activity of other antifolates including methotrexate, trimethoxetate, and lometrexol (7–10). These studies found more dramatic differences in drug activities with the absence or presence of MTAP; however, comparisons were made between nonsyngeneic cells that may have been complicated by differences in other factors that influence antifolate activity (7, 10). Likewise, the relevance of MTAP overexpression produced by the transfection of MTAP in cells, to the effect of constitutive levels of enzyme activity is not clear (8, 9). Suppression of constitutive MTAP activity was evaluated in one study with 5′-chloro-5′-deoxyformycin A (Kᵣ = 0.3 μmol/L; ref. 7). This agent resulted in a >25-fold decrease in the methotrexate IC₅₀ in the presence of thymidine. It is possible that the greater effect of MTAP suppression on methotrexate activity is due to the fact that this enzyme salvages not only adenine but methionine as well from methylthioadenosine. Unlike pemetrexed, which acts by direct suppression of tetrahydrofolate-dependent enzymes and does not deplete cellular folates (20), methotrexate depletes cells of tetrahydrofolate cofactors, including 5-methyltetrahydrofolate which is required for methionine synthesis (20, 21). Hence, when the MTAP inhibitor is coadministered with methotrexate, there are two factors that limit the availability of methionine. A possible role for methionine is further supported by the observation that differences in the methotrexate IC₅₀ between MTAP(+) and MTAP(−) cells are greater in methionine-free (6.2-fold) than methionine-containing (3.5-fold) media (8).

MTAP deletions have been shown in a wide variety of cancers including T cell acute lymphoblastic leukemia (22), pancreatic cancer (23), osteosarcoma (24), biliary tract cancers (11), gliomas (25), and melanoma (14). More relevant to pemetrexed, MTAP is deleted in ~70% of pancreatic mesotheliomas (4) and in ~38% of non–small cell lung cancers (26). This deletion has been exploited in an attempt to develop targeted chemotherapy regimens mainly focusing on using L-alanosine, an inhibitor of adenylsucci

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