Synergistic Activity of 5-Trifluoromethylthioribose and Inhibitors of Methionine Synthesis against Klebsiella pneumoniae

PAULA A. TOWER,1,2 LINDA L. JOHNSON,1 ADOLPH J. FERRO,3 JOHN H. FITCHEN,1,2 AND MICHAEL K. RISCOE1,2,*

Medical Research Service, Department of Veterans Affairs Medical Center, 3710 S.W. U.S. Veterans Hospital Road,1,2 and Department of Medicine, Oregon Health Sciences University,3 Portland, Oregon 97201, and Epitope, Inc., Beaverton, Oregon 97006

Received 14 June 1990/Accepted 20 October 1990

5-Methylthioribose (MTR) is an intermediate in the methionine recycling pathway of organisms containing the enzyme MTR kinase. Analogs of MTR have been proposed as a new class of antimicrobial agents because of their ability to perturb the growth of MTR kinase-containing pathogens through inhibition of methionine salvage or by conversion to toxic products. One such analog, 5-trifluoromethylthioribose (TFMTR), has demonstrated potent inhibitory effects on the growth of Klebsiella pneumoniae (A. G. Gianotti, P. A. Tower, J. H. Sheley, P. A. Conte, C. Spiro, J. H. Fitchen, and M. K. Riscoe, J. Biol. Chem. 265:831–837, 1990). Although the mode of action of TFMTR has yet to be determined, it is believed that the drug is converted to the toxic products trifluoromethionine or carbonothioic difluoride via MTR kinase and the methionine recycling pathway. On the basis of this assumption, we theorized that blocking de novo methionine synthesis would increase dependence on the methionine salvage pathway and lead to an increased rate of synthesis of toxic metabolites from TFMTR. In this report, we show that three separate inhibitors of de novo methionine synthesis (1,2,4-triazole, azaserine, and propargylglycine) act synergistically with TFMTR in inhibiting the growth of K. pneumoniae.

The importance of methionine to cell function is indicated by its critical role in protein synthesis, S-adenosylmethionine-dependent methyl transfer reactions, and polyamine biosynthesis (8). The demands upon the methionine pool from these processes make salvage of this energetically "expensive" amino acid advantageous for cellular function. One such salvage pathway is the remethylation of homocysteine in a process which involves vitamin B12 and 5-methyltetrahydrofolate (4). Another methionine salvage pathway involves the recycling of methionine from 5-methylthioadenosine (MTA), which is important for conserving the amino acid during the synthesis of the polyamines spermidine and spermine (Fig. 1) (29, 33).

Two distinct mechanisms exist for the salvage of methionine from MTA. In mammalian cells (2, 3) and some microorganisms (31), MTA is degraded in one step to adenine and 5-methylthioribose-1-phosphate (MTR-1-P) by MTA phosphorlylase (26). In other microbes (including some members of the family Enterobacteriaceae), MTA is catabolized in two steps: first to adenine and MTR via MTA nucleosidase (9), followed by conversion of MTR to MTR-1-P via MTR kinase (10). In both cases, MTR-1-P is synthesized, but by two distinct metabolic pathways. In many procaryotic and eucaryotic cells, MTR-1-P is then recycled to methionine via the intermediates 1-phospho-5-S-methylthiobulose (13, 32), 1-phospho-2,3-diketo-5-S-methylthiopentane (12), and 2-keto-4-methylthiobutyrate (1, 30).

Since MTR kinase is not present in human cells and because it is important in microbial methionine recycling, it is a logical target for chemotherapeutic drug design (27, 28). In a recent study, we showed that 5-trifluoromethylthioribose (TFMTR), a structural analog of MTR, exerts potent inhibitory effects on Klebsiella pneumoniae, an MTR kinase-containing organism (11, 14). Although the mode of action of TFMTR against K. pneumoniae has not been delineated, it is believed that TFMTR is converted within organisms to a toxic product (e.g., trifluoromethionine or carbonothioic difluoride) via the methionine salvage pathway. If this is the case, one might expect inhibition of de novo synthesis of methionine to enhance the inhibitory activity of TFMTR toward MTR kinase-containing microbes. Presumably, inhibition of de novo methionine synthesis would increase reliance on the methionine salvage pathway for maintenance of methionine levels, thereby leading to increased conversion of TFMTR to toxic products. In order to investigate this possibility and to gain insight into the mode of action of TFMTR, we examined the effect of pairing TFMTR with three inhibitors of microbial methionine synthesis. These compounds (1,2,4-triazole, azaserine, and propargylglycine) interfere with methionine synthesis through distinctly different mechanisms (Fig. 1) (20, 23, 25). In this report, we show that these agents act synergistically with TFMTR in inhibiting the growth of K. pneumoniae.

MATERIALS AND METHODS

Chemicals. Azaserine (O-diazoacetylserine), DL-propargylglycine, and 1,2,4-triazole were purchased from Sigma Chemical Co. (St. Louis, Mo.). TFMTR was synthesized as described previously (14).

Bacterial strains and culture conditions. A clinical isolate of K. pneumoniae, obtained from the Department of Veterans Affairs Medical Center, Portland, Oreg. (Clinical Diagnostic Laboratory, David Sewell), was utilized in this study. The identity of the isolate was verified with a standard microbiological diagnostic kit (Micro-ID, Organon Teknika, Durham, N.C.). K. pneumoniae was maintained in a chemically defined medium (21) containing 25 mM NH4Cl, 35 mM glucose, 1.5 mM KCl, 0.4 mM MgSO4, 0.045 mM NaCl, 1557
FIG. 1. Biosynthesis of cysteine and methionine in enteric bacteria and rationale for potentiation of TFMTR by 1,2,4-triazole, azaserine, and propargylglycine.
0.025 mM FeSO₄, 0.025 μg of thiamine per ml, and 66.6 mM Na₂HPO₄-NaH₂PO₄. The following micronutrient solution was added with the indicated final concentrations: CaCl₂ (5 × 10⁻⁷ M), CoCl₂ (5 × 10⁻⁸ M), MnCl₂ (10⁻⁷ M), H₂BO₃ (5 × 10⁻⁷ M), ZnCl₂ (10⁻⁸ M), CuCO₃ (10⁻⁸ M), (NH₄)₂MoO₄ (5 × 10⁻⁸ M); the medium was adjusted to pH 7.2. Dose inhibition studies were conducted in 5-ml cultures inoculated with ~10⁶ cells per ml and maintained in a rotary shaker incubator at 37°C. Growth was monitored by optical density at 470 nm 12 to 15 h after incubation or after control cultures reached an optical density of ~0.4. Isoboles representing the activity of drug mixtures were performed and analyzed as described by Hewlett (16). Briefly, TFMTR and 1,2,4-triazole, azaserine, or propargylglycine were serially diluted so that the organisms were simultaneously exposed to drug mixtures. The abilities of the various drug combinations to inhibit growth by 50% (IC₅₀) relative to control values were measured. When combined and plotted graphically, the results yielded an isobologram. As described by Hewlett, isoboles for two separately active drugs resemble a straight line for additive action, a convex line for subadditive action, and a concave line for potentiation (16).

RESULTS

Effect of methionine on the inhibitory action of TFMTR. In order to gain insight into the mechanism of action of TFMTR, we tested the ability of methionine to reverse the inhibitory effect of TFMTR. K. pneumoniae was cultured in defined medium containing 1 μM TFMTR and different amounts of methionine. The organisms were inoculated at a density of ~10⁶/ml and incubated for 12 to 15 h at 37°C. In the absence of added methionine, TFMTR totally inhibited cell growth. Less than 10% of control values was observed when methionine was added at 10 μM in the presence of TFMTR. Increasing the concentration of methionine to 100 μM restored growth to nearly 60% of control. The inhibitory action of TFMTR was completely abrogated by the addition of 1,000 μM methionine. Other amino acids, such as glycine, isoleucine, serine, threonine, aspartic acid, glutamic acid, and glutamine, had no effect on the antimicrobial action of TFMTR (data not shown). The fact that methionine reverses the antibacterial activity of TFMTR is consistent with the theory that TFMTR blocks cell growth by exploiting methionine recycling. Furthermore, this finding suggests that blocking microbial methionine biosynthesis will enhance the inhibitory action of TFMTR.

Synergy between TFMTR and inhibitors of methionine biosynthesis. 1,2,4-Triazole is an inhibitor of O-acetylserine sulfhydrylase, the final step in microbial cysteine biosynthesis (Fig. 1) (25). Accordingly, the antimicrobial effect of 1,2,4-triazole can be reversed by the addition of cysteine but not cystathionine or homocysteine (25 and unpublished data). We tested the ability of 1,2,4-triazole to augment the antimicrobial activity of TFMTR. As described by Gianotti et al. (14), the clinical strain of K. pneumoniae used in our studies contains MTR kinase, actively salvages methionine from MTA in vivo, and is very sensitive to the effects of TFMTR, exhibiting an IC₅₀ in the submicromolar range. As shown in Fig. 2A, 1,2,4-triazole is a weak but effective inhibitor of bacterial growth with an IC₅₀ of 2.5 mM. The concave line of the isobologram drawn from the collected IC₅₀'s indicates synergy between the two drugs. In combination, 0.1 μM TFMTR decreased the IC₅₀ for 1,2,4-triazole by 10-fold to 0.25 mM. The degree of potentiation measured as

FIG. 2. Graphic representation of synergy between TFMTR and various inhibitors of microbial methionine biosynthesis: 1,2,4-triazole (A), azaserine (B), and propargylglycine (C). The isobolograms are drawn with the IC₅₀ for K. pneumoniae on an arithmetic scale. Points reflect the mean of three separate experiments, the results of which did not differ by more than 15%.
the joint action ratio for the drug combination was calculated to be 2.5.

Azaserine is a substrate for O-acetylserine sulphydrylase (Fig. 1). Upon reaction of azaserine with this enzyme, diazoacetate, a highly reactive and toxic product, is formed (23). Unlike 1,2,4-triazole, cysteine is unable to completely reverse the inhibitory effect of azaserine (unpublished data), presumably because of the production of toxic diazoacetate. Studies with TFMTR and azaserine demonstrated a striking synergy (Fig. 2B). The IC\textsubscript{50} for azaserine alone against K. pneumoniae was 2.0 \textmu M. At all drug ratios, the amount of either drug required to produce 50\% inhibition was less with the combination than with either drug alone and became minimal when 0.05 \textmu M TFMTR was combined with 0.25 \textmu M azaserine. The degree of potentiation for TFMTR and azaserine measured as the joint action ratio was 3.6.

Propargylglycine is an irreversible inhibitor of cystathionine \gamma-synthase (20), a pyridoxal phosphate-dependent enzyme involved in microbial methionine synthesis (19) (Fig. 1). The growth inhibitory effects of propargylglycine are reversed by methionine (7, 20). In our studies, -375 \textmu M propargylglycine was required to inhibit \textit{Klebsiella} growth by 50\%. As shown in Fig. 2C, combining as little as 0.1 \textmu M TFMTR with 20 \textmu M propargylglycine produced the same degree of growth inhibition. All points from the TFMTR-propargylglycine combinations used in the study fell well below the line of addition. The degree of potentiation between the two drugs was calculated to be 3.2.

**DISCUSSION**

Microbial biosynthesis of cysteine and methionine proceeds along a branched convergent pathway in one arm of which sulfate is reduced to sulfide while serine is acetylated to O-acetylserine (Fig. 1) (19, 23). The final step consists of formation of cysteine from sulfide and O-acetylserine by the enzyme O-acetylserine sulphydrylase (24). Methionine is then synthesized from cysteine via cystathionine in a sequence of reactions catalyzed by cystathionine \gamma-synthase (22), cystathionine \beta-lyase (15), and methionine synthase (4). Thus, unlike mammalian cells, which synthesize cysteine from methionine, enteric bacteria derive methionine from cysteine (19).

In this study, we employed three different agents which perturb microbial cysteine and methionine biosynthesis to test the ability of these compounds to act in synergy with TFMTR. 1,2,4-Triazole blocks cysteine and methionine production through competitive inhibition of O-acetylserine sulphydrylase (23), while azaserine (O-diazoacylserine) serves as an alternate substrate for the enzyme, giving cysteine and toxic diazoacetate as products. Propargylglycine blocks methionine biosynthesis through irreversible inactivation of cystathionine \gamma-synthase (20). Our results show that all three compounds act synergistically with TFMTR to inhibit the growth of \textit{K. pneumoniae}. In fact, the degree of synergy between TFMTR and one of the compounds tested (azaserine) approaches that observed between trimethoprim and the sulfonamides (6). Taken together, these findings are consistent with the proposed modes of action of TFMTR, e.g., competitive inhibition of MTR kinase and/or conversion to a toxic product via the methionine recycling pathway.

A diverse group of drugs may similarly augment the action of TFMTR. For example, trimethoprim (a folate antagonist) acts to inhibit dihydrofolate reductase, resulting in a general depletion of reduced folate cofactors needed for synthesis of purines and thymidine as well as methionine (17, 18). It is conceivable that the block in methionine production exerted by trimethoprim will potentiate TFMTR in a manner that is analogous to that of the drugs tested in this report. Inhibitors of polyamine biosynthesis (5) may also be useful in combination with TFMTR by virtue of their ability to block production of MTR (Fig. 1). Theoretically, polyamine antagonists which block synthesis of spermidine and spermine will decrease intracellular levels of MTR and allow a higher rate of conversion of TFMTR to toxic products. Future work in this laboratory will test these possible drug combinations.

Chemotherapy employing multiple drugs with different modes of action is important to prevent the emergence of drug-resistant pathogens. Ideally, such drugs should act synergistically toward the elimination of the invading parasite. From the results presented here, it is clear that compounds which block microbial methionine metabolism act synergistically with TFMTR. Furthermore, the combination of TFMTR with an antagonist of microbial methionine synthesis provides a novel approach towards the control of infections caused by MTR kinase-containing pathogens.

**REFERENCES**

This investigation received financial support from the Department of Veterans Affairs Merit Review Program. Additional support was from Epitope, Inc., of Beaverton, Ore., and SmithKline Beecham Pharmaceuticals of Welwyn Garden City, United Kingdom.

We also thank David Peyton (Portland State University) for nuclear magnetic resonance analysis of MTR analogs.


12. Furfine, E. S., and R. H. Abeles. 1988. Intermediates in the conversion of 5'-S-methylthioadenosine to methionine in Kleb-


