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Antifolate Activity of Epigallocatechin Gallate against *Stenotrophomonas maltophilia*

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The catechin epigallocatechin gallate, one of the main constituents of green tea, showed strong antibiotic activity against 18 isolates of *Stenotrophomonas maltophilia* (MIC range, 4 to 256 μg/ml). In elucidating its mechanism of action, we have shown that epigallocatechin gallate is an efficient inhibitor of *S. maltophilia* dihydrofolate reductase, a strategic enzyme that is considered an attractive target for the development of antibacterial agents. The inhibition of *S. maltophilia* dihydrofolate reductase by this tea compound was studied and compared with the mechanism of a nonclassical antifolate compound, trimethoprim. Investigation of dihydrofolate reductase was undertaken with both a trimethoprim-susceptible *S. maltophilia* isolate and an isolate with a high level of resistance. The enzymes were purified using ammonium sulfate precipitation, gel filtration, and methotrexate affinity chromatography. The two isolates showed similar levels of dihydrofolate reductase expression and similar substrate kinetics. However, the dihydrofolate reductase from the trimethoprim-resistant isolate demonstrated decreased susceptibility to inhibition by trimethoprim and epigallocatechin gallate. As with other antifolates, the action of epigallocatechin gallate was synergistic with that of sulfamethoxazole, a drug that blocks folic acid metabolism in bacteria, and the inhibition of bacterial growth was attenuated by including leucovorin in the growth medium. We conclude that the mechanism of action of epigallocatechin gallate on *S. maltophilia* is related to its antifolate activity.

Stenotrophomonas maltophilia has emerged as an important nosocomial pathogen, especially for patients whose immune systems are compromised by debilitating diseases, and is associated with increasing case/fatality ratios. The major risk factors for *S. maltophilia* infection include long-term hospitalization, previous antimicrobial therapy, fungal infections, catheterization, and mechanical ventilation. *S. maltophilia* infection can cause bacteremia, endocarditis, pneumonia, mastoiditis, peritonitis, meningitis, or infections of the eyes, bones, joints, urinary tract, soft tissues, and wounds (4, 7, 10, 19, 21, 29, 30, 39). The management of infections caused by *S. maltophilia* is particularly difficult because of its inherent resistance to many currently available broad-spectrum antibiotics (5, 10, 11, 20, 22, 34).

The treatment of choice for *S. maltophilia* infection is trimethoprim-sulfamethoxazole (TMP-SMZ; cotrimoxazole), alone or in combination with ticarcillin-clavulanate (25, 32, 33). TMP-SMZ is bacteriostatic for most isolates; hence, high doses (12 to 15 mg/kg of body weight/day based on TMP) are usually recommended. Both drugs block folic acid metabolism in bacteria and are much more active together than either agent is alone. Sulfonamides are competitive inhibitors of the incorporation of *p*-aminobenzoic acid, while TMP is an inhibitor of the dihydrofolate reductase (DHFR; 5,6,7,8-tetrahydrofolate:NADP$^+$ oxidoreductase; EC 1.5.1.3) reaction. It is well known that DHFR catalyzes the NADPH-dependent reduction of 7,8-dihydrofolate (DHF) to 5,6,7,8-tetrahydrofolate (THF), which acts as a coenzyme for a number of 1-carbon transfer reactions, including those involved in nucleotide biosynthesis. Consequently, inhibition of DHFR leads to the disruption of DNA synthesis; this is the basis of the antibiotic action of DHFR inhibitors, the antifolates (13). Although TMP is currently used for the treatment of *S. maltophilia* infections, the mechanism by which this compound inhibits *S. maltophilia* DHFR has not been well characterized. Therefore, in this study we purified the DHFR from this microorganism for the first time, and we present data on its inhibition by classical (methotrexate [MTX]) and nonclassical (TMP) antifolate compounds.

Recent studies have presented data on a number of biological activities of tea polyphenols, or catechins (14, 23, 26). It has been reported that tea catechins have antibacterial activity against various pathogenic bacteria (15, 16, 23, 37). There are three main varieties of tea, green, black, and oolong, which are all derived from the leaves of the *Camellia sinensis* plant. The difference between the teas results from their processing. Green tea is prepared from unfermented leaves, the leaves of oolong tea are partially fermented, and black tea is fully fermented. This difference in processing results in more of the polyphenols being destroyed in the black teas. Thus, green tea contains roughly 30% to 40% polyphenols, while black tea contains only 3% to 10%. Green tea, therefore, seems to have more of the beneficial effects mentioned above, but black teas still retain some of the benefits. Epigallocatechin gal-

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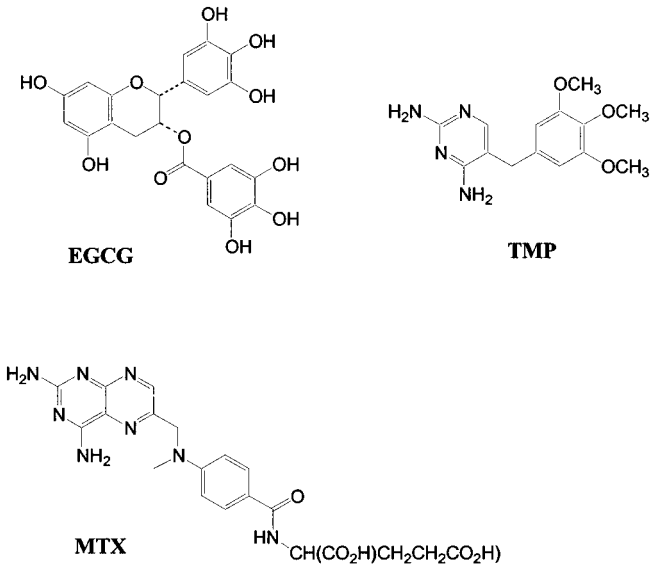

FIG. 1. Structural formulae of (−)-epigallocatechin gallate, TMP, and MTX.

late (EGCG) is the most abundant of these tea catechins (one 240-ml cup of brewed green tea contains up to 200 mg EGCG), and many health-related benefits, including antioxidant, antibiotic, and antiviral activities, have been attributed to this compound (26). Despite the great efforts made during the last 2 decades to understand the biological activity of tea, the exact mechanism(s) of action is not well defined. Therefore, deciphering the molecular mechanism by which green tea or EGCG exerts its antibacterial effects could be important because it may result in improved opportunities for the treatment of different bacterial infections. In attempting to explain the range of responses of *S. maltophilia* to tea phenols observed in our laboratory, we were struck by the structural similarity of EGCG to several inhibitors of DHFR, in particular, to the drugs MTX and TMP (Fig. 1). In order to probe the hypothesis that EGCG could act as an antifolate compound, we studied the inhibition of *S. maltophilia* DHFR by this tea compound and compared it with inhibition by TMP.

MATERIALS AND METHODS

Bacterial strains. Eighteen isolates of cotrimoxazole-susceptible *S. maltophilia* were collected over a period of 1 year at the Hospital Universitario Virgen de la Arrixaca (Murcia, Spain). Bacteria were frozen at −70°C in glycerol-meat medium and inoculated onto Columbia agar (Fluka Chemie GmbH, Madrid, Spain) supplemented with 5% defibrinated sheep blood 48 and 24 h prior to susceptibility testing.

EGCG and antibiotics. EGCG was obtained from Sigma Chemical Co. (Madrid, Spain). Stock dilutions were prepared in 0.15 mM H_3PO_4 to avoid oxidation of the drug. The other antibiotics (TMP and SMZ) were also obtained from Sigma. Stock dilutions of SMZ and TMP were prepared by following the National Committee for Clinical Laboratory Standards (NCCLS) guidelines (27).

Purification of DHFR. For the DHFR extraction, isolates 1 and 5 were inoculated onto MacConkey agar (Oxoid Ltd., Basingstoke, England) 24 h before use. Then liquid medium, Brilliant Green Bile 2% broth (Oxoid), was inoculated with the isolates, and these broth cultures were incubated aerobically at 37°C and shaken at 100 cycles per min. Bacteria were grown to mid-log phase, harvested by centrifugation (1,600 rpm, 30 min), and washed twice in 50 mM phosphate buffer (pH 7.0), followed each time by a new centrifugation (1,600 rpm, 5 min). The cell lysis, centrifugation, and dialysis steps were carried out between 4 and 8°C. Fast protein liquid chromatography purification steps were performed at room temperature. Cell paste from 2 liters of culture (approximately 10 g of bacteria) was suspended in 30 ml of buffer A (5 mM Tris-HCl, pH 7.4, 1 mM EDTA) containing 0.1 mM phenylmethylsulfonyl fluoride as a protease inhibitor, and the cell suspension was homogenized in a Potter homogenizer, followed by ultrasonication. After centrifugation at 36,000 rpm for 30 min to remove cell debris, the supernatant was filtered. This supernatant was brought to 40% saturation with solid ammonium sulfate under continuous stirring. After 1 h the solution was centrifuged at 35,000 rpm for 30 min, and the pellet was discarded. Additional ammonium sulfate was added to the clear supernatant to give 90% saturation, and the mixture was stirred for 1 h. After centrifugation, the precipitates were suspended in 2 ml of buffer B (10 mM potassium phosphate buffer, pH 7.4, 2 mM β-mercaptoethanol). Concentrated enzyme (2-ml samples) was loaded onto a gel filtration column (Sephacryl S-75 26/60 Hi-Prep; Amersham Pharmacia Biotech Europe GmbH, Barcelona, Spain) equilibrated with buffer B and eluted at 0.5 ml/min. The active fractions were applied to an MTX-agarose (Sigma) column equilibrated with 50 mM potassium phosphate buffer, pH 6.5, containing 100 mM KCl. The column was then washed with 200 ml 50 mM potassium phosphate buffer, pH 6.5, containing 2 M KCl. The enzyme was eluted using 10 ml of 50 mM Tris-HCl, pH 8.6, containing 1 M KCl and 2 mM folic acid. Fractions containing DHFR activity were combined, dialyzed overnight against 2 liters buffer B (three times), concentrated in an Amicon concentrator (YM-10 membrane), and stored at −80°C. The DHFR concentration was determined by MTX titration of enzyme activity (35), while the total protein concentration was determined using the Bio-Rad protein assay procedure with bovine serum albumin as the standard.

DHFR assays and kinetics data analysis. DHF was obtained from Aldrich Chemie GmbH (Madrid, Spain) and NADPH from Sigma. DHFR activity was determined at 25°C by monitoring the decrease in absorbance of NADPH and DHF at 340 nm (ε = 11,800 M^{-1} cm^{-1} [38]) using a Perkin-Elmer Lambda-2 spectrophotometer with 1.0-cm-light-path cuvettes. Temperature was controlled at 25°C using a Haake D1G circulating bath with a heater/cooler and was checked using a Cole-Parmer digital thermometer with a precision of ±0.1°C. Experiments were performed in a buffer containing 2-(*N*-morpholino)ethanesulfonic acid (MES; 25 mM), sodium acetate (25 mM), Tris (50 mM), and NaCl (100 mM). The pH of the reaction was measured before and after the experiment. The assays were started by adding the enzyme. In the absence of the enzyme, the rate of absorbance change was negligible. The concentrations of DHFR, NADPH, and DHF are given in the text or in the legends to the figures. One unit is defined as the amount of enzyme required to convert 1 μmol of DHF to THF in 1 min at 25°C. The maximum steady-state rate (V_{max}) and the Michaelis constants of DHFR for DHF (K_m^{DHF}) and NADPH (K_m^{NADPH}) were determined from the curvature evident in plots of disappearance of NADPH and DHF versus time (10 determinations). For K_m^{DHF} or K_m^{NADPH} determinations, the initial concentration of saturating NADPH (100 μM) or DHF (200 μM) was considered constant during the overall consumption of 10 μM DHF or 20 μM NADPH by the enzyme (3 nM), respectively. Data were fitted by nonlinear regression to the integrated form of the Michaelis equation (6), using Marquardt's algorithm (24) implemented in Sigma Plot 8.02 for Windows (36).

DHFR inhibition experiments and kinetics data analysis. Initial velocity inhibition experiments were carried out on *S. maltophilia* DHFR with TMP and EGCG. For this purpose, one substrate (NADPH) was held constant at the saturating concentration while the other substrate (DHF) and the inhibitor (TMP or EGCG) were varied. To prevent the oxidation of EGCG, the reaction mixture contained 1 mM *N*-acetylcysteine (Sigma). The extent of recovery of enzymatic activity following inhibition induced by preincubation with DHFR inhibitors was determined as follows. DHFR (0.15 μM) was preincubated for 30 min at 25°C in a buffer containing TMP or EGCG. An aliquot of the incubation mixture was then diluted 50-fold into a reaction mixture containing the buffer, NADPH (100 μM), and DHF (20 μM). The recovery of enzyme activity was determined by continuous monitoring at 340 nm.

Broth dilution MIC determination. MICs for the 18 isolates were determined by the broth dilution method at a final inoculum of 5×10^5 CFU/ml using cation-adjusted Mueller-Hinton broth (Fluka). The final inoculum was verified by plating in duplicate 100 μl of a 100-fold saline dilution onto MacConkey agar according to the NCCLS guidelines (27). After aerobic incubation at 35°C for 24 h, the lowest concentration of the twofold serially diluted antibiotic at which no visible growth occurred was defined as its MIC.

Time-kill assays for detection of EGCG bactericidal and bacteriostatic effects. A time-kill assay was performed for isolate 1. Glass tubes containing cation-adjusted Mueller-Hinton broth, with doubling antibiotic concentrations, were inoculated with 5×10^5 CFU/ml and were incubated aerobically at 35°C for 24 h. Antibiotic concentrations were chosen to comprise three twofold concentrations above and two twofold dilutions below the broth dilution MIC. Inoculation of

TABLE 1. Susceptibilities of *S. maltophilia* to tested antibiotics

Isolate no.	Susceptibility (MIC [μg/ml]) to:			
	TMP	EGCG	SMZ	SXT[a]
7	4	4	0.5	0.25
18	4	8	16	0.125
5	4	16	8	0.19
13	4	32	0.5	0.25
3	4	64	4	0.014
8	4	128	8	0.094
17	8	32	8	0.25
10	8	64	4	0.19
12	8	64	8	0.094
6	8	128	32	0.25
11	16	32	16	0.5
14	16	32	16	0.25
9	16	256	8	0.19
2	32	64	16	0.38
4	32	64	64	0.25
16	64	32	128	2
15	64	64	64	0.5
1	128	64	64	1

[a] For cotrimoxazole (SXT), the MICs refer to the TMP component.

each serially diluted antibiotic tube was performed by following NCCLS guidelines for the broth dilution method (27). Viability counts of antibiotic-containing suspensions were carried out at 0, 3, 6, 12, and 24 h by plating 10-μl aliquots of 10-fold dilutions from each tube in sterile saline onto Columbia agar supplemented with 5% defibrinated sheep blood. The plates used to recover organisms were incubated at 37°C for 24 h. The lower limit of sensitivity of colony counts was 100 CFU/ml. Time-kill assay results were analyzed by determining changes in the $\log_{10}$ CFU/ml compared to the counts at time zero for the six different concentrations of EGCG. Bactericidal activity was defined as a reduction from the count in the initial suspension of $\geq 3 \log_{10}$ CFU/ml after incubation at 37°C for 24 h, while the effect was considered bacteriostatic if the inoculum was reduced by 0 to 3 $\log_{10}$ CFU/ml.

Checkerboard synergy testing. Checkerboard tests were performed for all isolates by broth dilution in cation-adjusted Mueller-Hinton broth combining eight doubling concentrations of EGCG with another eight dilutions of SMZ and TMP, respectively. The inoculum was prepared by suspending bacterial growth from blood agar plates in sterile saline to a final density of 0.5 McFarland and diluting in Mueller-Hinton broth to a final inoculum of 5×10^5 CFU/ml. Tubes were incubated aerobically overnight at 35°C. Fractional inhibitory combinations (FICs) were calculated as the MIC of the antibiotic and EGCG in combination divided by the MIC of the antibiotic or EGCG alone, and the FIC index was obtained by adding the FICs. FIC indices were defined as synergistic when values were ≤ 0.5 and antagonistic when values were >4. Results that fell between synergy and antagonism were defined as additive or indifferent.

Time-kill synergy determinations. Time-kill tests were performed in glass tubes with 1 ml of cation-adjusted Mueller-Hinton broth. Each isolate was tested against TMP, SMZ, and EGCG alone at a concentration equivalent to 0.25 times the MIC (0.25×MIC) and in combinations at this concentration. Antimicrobial solutions were transferred to the tubes and inoculated with each isolate. The final inoculum was approximately 5×10^5 CFU/ml. Tubes were incubated aerobically at 35°C for 16 h. Colony counts were performed at 0 and 16 h. Samples (100 μl) from these tubes were removed and inoculated into serial 10-fold dilutions in 0.9% saline. Aliquots (50 μl) were then placed on MacConkey agar plates for counting of surviving colonies. The limit of quantification was of 5×10^2 CFU/ml. Synergy was assumed when a $\geq 2 \log_{10}$ decrease in the viable colony count was obtained with the combination at 24 h, compared with the viable count obtained with the more active of the two compounds alone, and when a $\geq 2 \log_{10}$ decrease in the colony count was obtained with the combination at 24 h, compared with the starting inoculum.

Experiments with leucovorin. The 18 isolates (final inoculum, 5×10^5 CFU/ml) were grown on 96-well microplates containing TMP, SMZ, and EGCG (at 0.5×MIC for each isolate) in cation-adjusted Mueller-Hinton broth with and without 0.4 mM leucovorin (Sigma). Control plates contained no antibiotic. The plates were sealed and incubated aerobically at 35°C for 24 h. After that time, absorbance at 405 nm was read in a microplate spectrophotometer (SPECTRAmax, 340PC[384]; Molecular Devices Corporation, CA).

RESULTS

Antibacterial action of EGCG on *S. maltophilia*. The MICs of EGCG against 18 *S. maltophilia* isolates ranged from 4 to 256 μg/ml (Table 1). The bactericidal action of EGCG was also examined. Figure 2 shows representative data obtained with strain 1 exposed to 16, 32, 64 (MIC), 128, 256, and 512 μg of EGCG per ml. Bacteriostatic and bactericidal effects were observed after 12 h at 2×MIC and 4×MIC, respectively. Regrowth was observed with EGCG (64 μg/ml) after 12 h of incubation. Although the antibacterial mechanism of EGCG is still obscure, several studies have indicated that this activity is closely related to the gallic acid moiety and the number of hydroxyl groups (16). Recently, we have shown that ester-bonded gallate catechins isolated from green tea, such as EGCG and epicatechin gallate (ECG), are potent inhibitors of DHFR activity in vitro (28) at concentrations found in the sera and tissues of green tea drinkers (0.1 to 1.0 μM) (41). EGCG exhibited kinetics characteristic of a slow-binding inhibitor of bovine liver DHFR but of a classical, reversible, competitive inhibitor with chicken liver DHFR. Structural modeling showed that EGCG can bind to human DHFR in an orientation similar to that observed for a number of structurally characterized DHFR inhibitor complexes. These results suggested that EGCG could act as an antifolate compound in the same way as TMP. To compare the antimicrobial activities of EGCG and TMP against *S. maltophilia*, the MICs of TMP were also determined (Table 1). TMP MICs ranged from 4 to 128 μg/ml. Isolate 1 showed the highest resistance to this drug. Comparison of TMP MICs with EGCG MICs showed that in general, the sensitivities of the bacteria to the two compounds were similar. However, more antibacterial activity (lower MICs) was found for TMP against the majority of the isolates (isolates 1 and 16 were the exceptions). To determine if the antimicrobial action of EGCG against *S. maltophilia* could be due to the inhibition of DHFR, we have purified this enzyme

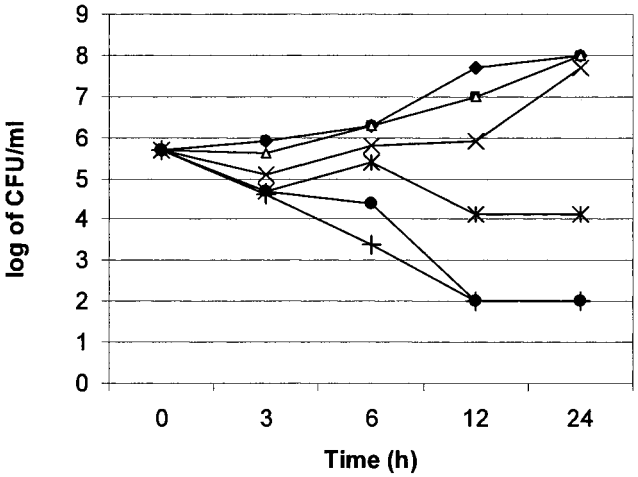

FIG. 2. Effect of EGCG on the viability of *S. maltophilia* isolate 1 in liquid medium (time-kill curve). *S. maltophilia* isolate 1 was cultured aerobically in cation-adjusted Mueller-Hinton broth at 35°C with reciprocation in the presence of EGCG at concentrations of 512 (+), 256 (●), 128 (✳), 64 (×), 32 (△), 16 (□), and 0 (◆) μg/ml. Culture samples (100 μl) were taken at the times indicated, and viability was measured by the plate colony count technique.

TABLE 2. Kinetics and inhibition characteristics of *S. maltophilia* DHFRs purified from isolates 1 and 5

Isolate	k_{cat} (s^{-1})	K_m^{NADPH} (μM)	K_m^{DHF} (μM)	K_i^{TMP} (μM)	MIC^{TMP} (μg/ml)	K_i^{EGCG} (μM)	MIC^{EGCG} (μg/ml)
1	2.1 ± 0.2	12 ± 3	1.8 ± 0.4	4.7 ± 0.1	128	4.0 ± 0.1	64
5	2.9 ± 0.3	10 ± 3	2.0 ± 0.5	0.06 ± 0.02	4	0.3 ± 0.05	16

from two *S. maltophilia* isolates with different sensitivities to TMP: isolates 1 (MIC, 128 μg/ml) and 5 (MIC, 4 μg/ml). The enzymes were kinetically characterized with respect to their substrates, and their inhibition by EGCG in vitro was studied and compared to that obtained using TMP.

Kinetics and inhibition studies of *S. maltophilia* DHFR. DHFR was purified from a TMP-resistant and a TMP-susceptible isolate (isolates 1 and 5, respectively) using techniques including MTX affinity chromatography. The specific activities of the two enzymes were similar in crude extracts (0.14 U/mg of protein). The homogeneity of final preparations was demonstrated in determinations of DHFR concentration versus protein concentration. A comparison of the kinetic properties of the DHFRs isolated from the two strains is shown in Table 2. The calculated K_m and k_{cat} values at pH 7.4 were essentially the same for the enzymes from the two isolates and similar to those of DHFRs from other species (3, 40). *S. maltophilia* DHFR was strongly inhibited by MTX (inhibition constant [K_i], 18 pM for isolate 1), showing slow-binding inhibition (12) as described for the inhibition of DHFRs from other biological sources by this compound (38). However, because MTX is not used clinically in the treatment of *S. maltophilia*-related diseases, this inhibition has not been further characterized.

Figure 3 presents representative steady-state kinetics data showing inhibition by TMP of isolate 1 DHFR. Preincubation of the enzyme with TMP (20 to 50 μM) for 30 min, followed by a 500-fold dilution into the standard DHF/NADPH assay medium, did not produce any measurable inhibition. Thus, the inhibition shown in Fig. 3 must involve the reversible binding of TMP to *S. maltophilia* DHFR. The same type of TMP inhibition was observed for isolate 5 DHFR. The K_i values for TMP as a competitive inhibitor of DHFRs purified from *S. maltophilia* isolates 1 and 5 are shown in Table 2. Isolate 1, with a higher resistance to TMP, showed a higher K_i. Lower values for both the MIC and K_i were found for isolate 5, confirming the well-known mechanism of action of this antifolate drug. Similar results were obtained by comparing TMP-resistant and TMP-susceptible isolates of *Staphylococcus aureus* (8).

Isolate 1 (MIC, 64 μg/ml) was also found to be more resistant to EGCG than isolate 5 (MIC, 16 μg/ml). To determine if this difference in sensitivity was also related to differences in the susceptibility of DHFR to EGCG inhibition, a complete kinetics study was carried out. The results for the inhibition of DHFRs from the two *S. maltophilia* isolates by EGCG are summarized in Table 2. Preincubation experiments with the enzymes in the presence of different concentrations of EGCG did not show any effect on enzyme activity. However, EGCG affected the initial rate of DHFR reaction with its substrates, NADPH and DHF. Double-reciprocal plots at a saturating concentration of NADPH and variable concentrations of DHF and EGCG showed a set of straight lines, which intercept on the ordinate axis (Fig. 4) (isolate 1). These results are characteristic of reversible and competitive inhibition with respect to DHF, with calculated K_i values of 4.0 μM for isolate 1 and 0.3 μM for isolate 5 (Table 2). Therefore, the data indicated that EGCG could act as an antifolate compound, DHFR being the main target for its action.

Comparative activity of EGCG combined with other agents. The action of antifolate compounds as competitive inhibitors of DHF is highly dependent on the concentration of folate compounds inside cells. It has recently been determined that folate depletion increases the sensitivity of solid tumor cell lines to several antifolates (1). Therefore, if the antibiotic effects of EGCG against *S. maltophilia* are related to the inhibition of DHFR, a combination of this compound with a sul-

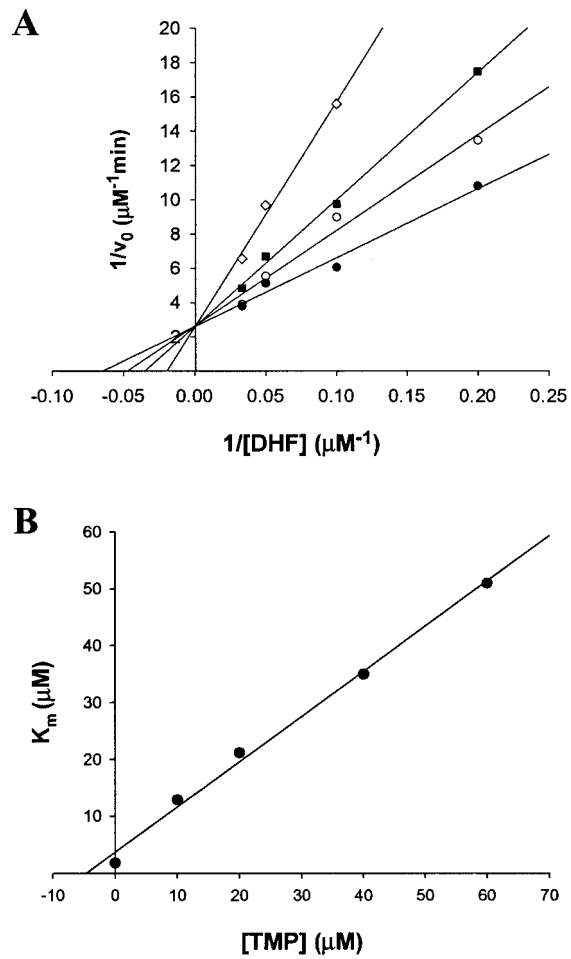

FIG. 3. (A) Double-reciprocal plots of the reaction of DHFR from *S. maltophilia* isolate 1 (3 nM) with NADPH (100 μM) and DHF (variable substrate) in the presence of TMP at pH 7.4. TMP concentrations were 10 (●), 20 (○), 40 (■), and 60 (◇) μM. Each point represents the mean ± standard deviation for five separate experiments. (B) Secondary plot for the apparent K_m^{DHF}, obtained from panel A, versus the concentration of TMP.

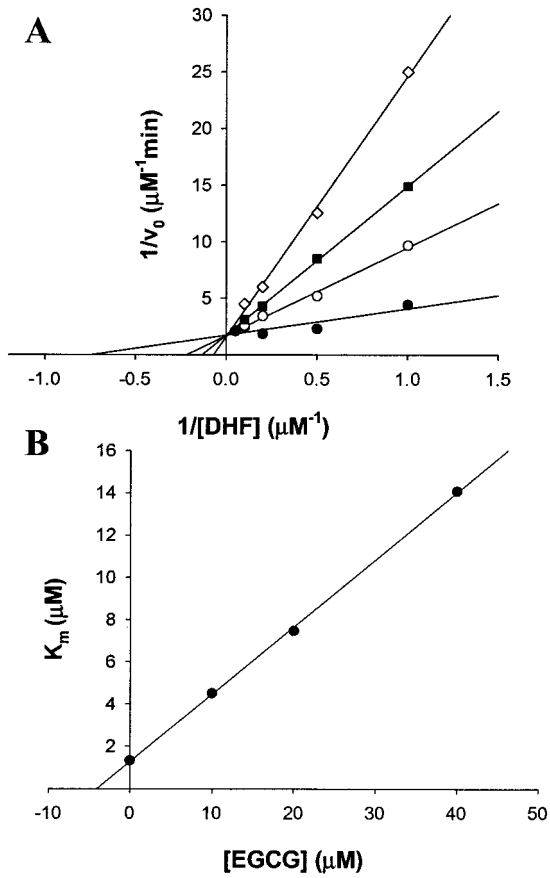

FIG. 4. (A) Double-reciprocal plots of the reaction of DHFR from *S. maltophilia* isolate 1 (3 nM) with NADPH (100 μM) and DHF (variable substrate) in the presence of EGCG at pH 7.4. EGCG concentrations were 0 (●), 10 (○), 20 (■), and 40 (◇) μM. Each point represents the mean ± standard deviation for five separate experiments. (B) Secondary plots for the apparent K_m^{DHF}, obtained from panel A, versus the concentration of EGCG.

fonamide (such as SMZ), an inhibitor of folic acid synthesis, should show synergy. This is the basis for the use of cotrimoxazole, which is the combination of an antifolate (TMP) and a sulfonamide, in the treatment of *S. maltophilia*-related diseases. Checkerboard titrations carried out in our laboratory revealed that 17 out of 18 *S. maltophilia* isolates tested showed synergy between EGCG and SMZ (Table 3). No FIC indices indicating antagonism were observed for any of the isolates. Similar experiments with EGCG and TMP showed no synergistic FIC indices (Table 3) (lowest FIC, 0.625); a simple additive effect was observed. These results indicate that EGCG could act as an inhibitor of DHFR and that sulfonamide could increase the antibiotic effect of EGCG by decreasing the folic acid levels in the bacteria.

A confirmation of the antifolate activity of EGCG against S. *maltophilia* was obtained from "rescue" experiments with folinic acid (leucovorin). Leucovorin is the active form of folic acid and is used as an antidote to drugs that decrease the levels of folic acid in the cells. Leucovorin is used in combination with TMP to prevent bone marrow toxicity and with methotrexate in cancer chemotherapy. Antifolates block the de novo biosynthesis of thymine, purines, and pyrimidines by inhibiting the synthesis of THF, an essential cofactor in these biosynthetic pathways. Bacterial cultures growing with leucovorin can increase their survival in the presence of antifolate compounds. Figure 5 shows the percentage of bacterial growth after 24 h of incubation with TMP, SMZ, or EGCG in the absence and presence of leucovorin. *S. maltophilia* grown in Mueller-Hinton medium enriched with 0.4 mM leucovorin showed a high level of inhibition reversal in the presence of EGCG or TMP. However, no reversal of growth inhibition was observed in the presence of SMZ. The data indicated that both EGCG and TMP could share the same mechanism of action as antifolate compounds.

TABLE 3. Synergy method results

Isolate no.	Checkerboard titration result				Time-kill assay result (interpretation)	
	SMZ/EGCG		TMP/EGCG			
	MIC (μg/ml)	FIC index (interpretation)[a]	MIC (μg/ml)	FIC index (interpretation)	0.25 × MIC SMZ + 0.25 × MIC EGCG	0.25 × MIC TMP + 0.25 × MIC EGCG
1	16/8	0.25 (S)	128/16	1.25 (A/I)	A/I	A/I
2	1/16	0.31 (S)	32/16	1.25 (A/I)	A/I	A/I
3	0.25/8	0.18 (S)	2/16	0.75 (A/I)	S	A/I
4	16/16	0.50 (S)	32/16	1.25 (A/I)	A/I	A/I
5	1/0.25	0.14 (S)	8/4	1.50 (A/I)	S	A/I
6	4/16	0.25 (S)	4/32	0.75 (A/I)	S	A/I
7	0.063/0.125	0.16 (S)	4/4	1.5 (A/I)	S	A/I
8	1/32	0.37 (S)	1/64	0.75 (A/I)	S	A/I
9	1/8	0.15 (S)	16/32	1.13 (A/I)	S	A/I
10	0.25/8	0.18 (S)	2/16	0.625 (A/I)	A/I	A/I
11	1/8	0.31 (S)	8/16	1.00 (A/I)	A/I	A/I
12	2/1	0.26 (S)	8/1	1.01 (A/I)	S	A/I
13	0.063/0.5	0.37 (S)	4/32	2.00 (A/I)	S	A/I
14	1/8	0.31 (S)	16/16	1.50 (A/I)	A/I	A/I
15	16/16	0.50 (S)	32/32	1.00 (A/I)	A/I	A/I
16	32/2	0.31 (S)	64/32	2.00 (A/I)	S	A/I
17	4/32	1.50 (A/I)	8/32	2.00 (A/I)	A/I	A/I
18	2/0.125	0.14 (S)	4/4	1.50 (A/I)	A/I	A/I

[a] S, synergy; A/I, antagonism/indifference.

DISCUSSION

Previously, EGCG has been shown to have strong antibacterial activity against several pathogenic microorganisms including *Helicobacter pylori* (23) and *Staphylococcus aureus* (15, 16). Here, we have observed for the first time that the gallated tea polyphenol EGCG exhibits antibiotic activity against *S. maltophilia*, an important nosocomial pathogen. To elucidate its mechanism of action, we have shown that EGCG acts as an effective inhibitor of S. *maltophilia* DHFR. The type of inhibition of *S. maltophilia* DHFR by EGCG (competitive with respect to DHF and reversible in a fast process) is similar to that of TMP but differs from that of MTX (competitive with respect to DHF; reversible slow binding). It has previously been observed that enzymes obtained from different sources differed with regard to their type of inhibition by MTX and other folate analogues (38). Thus, both MTX and TMP can be considered slow tight-binding inhibitors of the enzyme from *Escherichia coli*, but only MTX shows this type of inhibition with the chicken liver enzyme (38). Although the exact structural bases that determine the type of inhibition are not well understood, they might be conditioned by the interactions of the drugs with particular residues of the DHFR active site. Further experiments to determine the sequence and structure of this newly isolated DHFR are needed in order to better understand the basis of its interactions with antifolate compounds. MTX is a stronger inhibitor of *S. maltophilia* DHFR than EGCG or TMP. Although MTX is more active against *S. maltophilia*, its clinical use is precluded because it is also a strong inhibitor of human DHFR and therefore may cause many adverse side effects. However, EGCG could represent an alternative for use in *S. maltophilia* infections, especially for patients with a low tolerance of TMP. It has been observed that elderly patients with poor renal function find it difficult to tolerate high doses of TMP, which produces severe skin reactions, bone marrow suppression, and thrombocytopenia.

The findings of this study could be of interest because *S. maltophilia* is commonly resistant to many currently available broad-spectrum antimicrobial agents, including β-lactams, aminoglycosides, and quinolones (10). TMP alone or in combination with SMZ is an effective and inexpensive antibacterial remedy. Lately, however, a dramatic increase in TMP and SMZ resistance has been seen. Bacterial resistance to TMP and SMZ is mediated mainly by the following five mechanisms: (i) the permeability barrier and/or efflux pumps, (ii) naturally insensitive target enzymes, (iii) regulational changes in the target enzymes, (iv) mutational or recombinational changes in the target enzymes, and (v) acquired resistance by drug-resistant target enzymes. Naturally insensitive DHFR enzymes are found among, for instance, *Bacteroides* species, *Clostridium* species, *Neisseria* species, and *Moraxella catarrhalis* (17, 18). Overproduction of chromosomal DHFR caused by promoter mutations has reportedly occurred in *E. coli* (17). A single amino acid substitution in the DHFR gene and altered chromosomally encoded DHFR have been considered responsible for resistance to TMP in *S. aureus* (8) and *Streptococcus pneumoniae* (31). In strains of TMP-resistant *Haemophilus influenzae*, changes in both the promoter and coding regions of the DHFR genes have been found (9). From the beginning of the use of cotrimoxazole, approximately 20 different TMP-resistant transferable DHFR genes have been characterized (17). The most prevalent of these genes, the DHFRI gene and variants of the DHFRII gene, mediate high-level resistance to TMP, with MIC increases of more than 1,000-fold, and are more frequently found in gram-negative enteric bacteria. The resistance of *S. maltophilia* to TMP has not been well characterized, probably due to the lack of availability of its DHFR. Comparative studies of a highly TMP resistant *S. maltophilia* isolate (isolate 1) and a TMP-susceptible isolate (isolate 5) have allowed us to analyze the possible mechanisms of *S. maltophilia* TMP resistance. Although changes in the permeability barrier and/or efflux pumps of the bacteria for TMP could not be disregarded, the fact that DHFRs from isolates 1 and 5 showed a 67-fold difference in K_i (Table 2) indicates that the main mechanism of resistance for isolate 1 could well be related to changes in the target enzyme. The two isolates showed similar levels of DHFR expression; thus, mechanisms of resistance resulting from DHFR overexpression or changes in promoter and/or coding regions of the DHFR genes do not seem to be of importance. Therefore, resistance could be due to mutational changes in the gene for DHFR increasing the K_i for the drug or to the acquisition of drug-resistant target enzymes. However, the latter hypothesis is less probable, because an intensive study of *S. maltophilia* resistance to cotrimoxazole showed that it cannot be explained by the presence of plasmid-specified TMP resistance determinants and that the increase probably relies on a chromosomal mechanism (2).

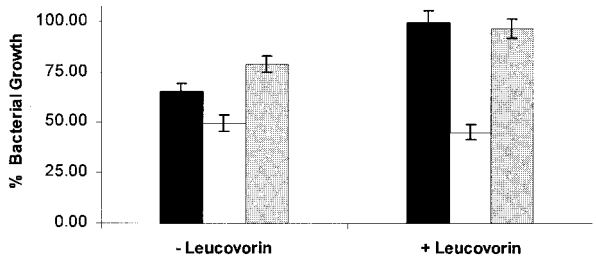

FIG. 5. Effects of TMP (black bar), SMZ (white bar), and EGCG (grey bar) on *S. maltophilia* growth after a 24-h incubation in the presence and absence of 0.4 mM leucovorin. The data are expressed assuming 100% growth for the untreated control. Bars represent the average growth for the 18 isolates, and the error bars represent the standard deviations of the data.

The antifolate character of EGCG is evident from its similarity to the action mechanism of the nonclassical antifolate TMP. Both inhibit S. *maltophilia* DHFR in a reversible, competitive, fast process and are synergistic with respect to SMZ, and bacterial growth inhibition in the presence of these drugs is reversed by growing S. *maltophilia* in a medium enriched with leucovorin. Although the MICs of TMP and EGCG are comparable, there is not an exact correlation between them for the different isolates studied, possibly indicating that different resistance mechanisms, not affecting DHFR, could also be involved. For example, factors related to permeability barriers and efflux pumps could differ for the drugs. The clinical relevance of these in vitro results will need to be confirmed by investigations of their therapeutic efficacy. Further studies with a higher number of TMP-resistant strains should be of interest to elucidate if EGCG maintains a good level of activity in all cases. If this proves to be the case, this drug could represent an

alternative to TMP and could be used in combination with SMZ for the treatment of S. *maltophilia* infections.

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