Sensitivity to Analogs of Thiamine Pyrimidine Associated with Resistance To Amethopterin or Purine Antagonists.*† (23090)

ROBERT GUTHRIE, MAUDE E. LOEBECK AND MARILYN J. HILLMAN
(Introduced by Erwin Neter)
Roswell Park Memorial Institute, N. Y. State Department of Health, Buffalo, N. Y.

Law(1) reported that certain lines of leukemia in mice became more sensitive to amethopterin when resistance to 6-mercaptopurine (6-MP) developed, or when resistant to, or "dependent" upon, 8-azaguanine (8-AG). Szybalski and Bryson(2) observed an instance of mutation in *Escherichia coli* wherein increased resistance to one antibiotic was associated with increased sensitivity to a second. They designated this phenomenon "collateral sensitivity." Subsequently, Elion, *et al.*(3) found a 2-6-diaminopurine-resistant strain of *Lactobacillus casei* that possessed increased sensitivity to 6-MP. Elion and Hitchings reported a 6-MP-resistant strain of *L. casei* with increased sensitivity to amethopterin, and conversely Hutchison and Burchenal(4) observed collateral sensitivity to 6-MP in an amethopterin-resistant *Streptococcus fecalis* strain. Schabel, Wheeler and Skipper reported that an azaserine-resistant strain of *E. coli* was more sensitive than the parent to 6-MP and diaminobiuret, and that a sulfanilamide-resistant strain showed collateral sensitivity to 6-chloropurine, 8-AG or purine.

The phenomena of collateral sensitivity and cross resistance have been used in this laboratory to screen compounds of potential interest in cancer chemotherapy. Since amethopterin and 6-MP are of interest in clinical acute leukemia, and resistance to them apparently arises in leukemic cells of rodents and humans, mutant organisms resistant to these two antimetabolites served as a "collateral sensitivity screen."

Materials and methods. Several amethopterin-resistant mutants were isolated from populations of the parent, *Bacillus subtilis* ATCC 6051, and from a purineless auxotroph, *B. subtilis* 6051-9. Mutants resistant to 6-MP were isolated from a purine-requiring *E. coli* strain, 9661-01 (P-)(5). From cultures of the 6-MP-resistant organisms, 2 mutant strains were isolated that were resistant simultaneously to 8-AG, 6-thioguanine (6-TG) and 6-MP. In all cases, drug-resistant mutants were isolated from solid agar media by incorporating large populations of the sensitive parent into minimal agar medium containing the drug, incubating, and sub-culturing discrete colonies that appeared. Using these resistant strains, 245 compounds were tested, including 175 pyrimidines, 22 purines, 5 sulfonamides, 2 antibiotics, 24 naturally occurring amino acids, azaserine and 16 miscellaneous compounds. Each compound was tested alone and also in combination with amethopterin or 6-MP, to detect possible synergistic effects. Any compound that produced growth-inhibition upon initial screening against resistant mutants was tested again upon the parent organism and the resistant mutant, under identical conditions, to compare sensitivity of parent and mutant. If the amethopterin- or 6-MP-resistant mutant consistently showed greater sensitivity to the test compound than the parent in 3 or more replicate experiments, the resistant strain was considered to possess collateral sensitivity to the test compound. The agar-diffusion method was used for routine testing. Two different minimal, chemically-defined agar media, one for each bacterial species(5) were homogeneously seeded with the test organism and distributed in Petri dishes. Each compound was tested by impregnating filter paper discs (12.7 mm diameter) with solutions containing approximately 0.5 μM and placing them upon the surface of the agar. The width of inhibitory zone, if any, as well as completeness of inhibition within the zone, was noted. In growth experiments with *B. sub-*

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TABLE I. Collateral Sensitivity to Amethopterin and to Certain Analogs of Thiamine Pyrimidine Exhibited by an *Escherichia coli* Mutant Resistant to 3 Purine Antagonists.* No inhibition was found in parent strain.

	Compound tested†		mm diam. inhibition zone
	R‡	X‡	Mutant
1.	HS–	–OH	0
2.	CH_3S–	–OH	34
3.	CH_3CH_2S–	–OH	0
4.	CH_3S–	$-OCH_2CH_3$	30
5.	CH_3CH_2S–	–Cl	34
6.	HO–	$-NH_2$	34
7.	Amethopterin		36

* 6-mercaptopurine, 8-azaguanine, 6-thioguanine.
† 0.05 cc of M/100 sol. impregnated in 12.7 mm paper disc, placed upon agar surface.
‡ 2-R-4-amino-5-Xmethyl pyrimidine.

tilis in liquid medium, agar was omitted but otherwise the same minimal medium was used. The medium was distributed in 6 cc amounts in culture tubes of convenient size (18 x 150 mm) for use in determination of optical density. These tubes were placed in a slanted position upon the platform of a rotary shaker. These conditions had been previously demonstrated to give adequate aerobiosis.

Results. In the course of testing growth inhibitory effects of 239 compounds against resistant mutants, only one compound, amethopterin, produced greater inhibition of a drug-resistant mutant than for the parent (collateral sensitivity). Two *E. coli* mutants, resistant to purine-antagonists, were more sensitive than the parent strain to amethopterin. This is shown for one mutant in Table I. Subsequently, a second case of collateral sensitivity was demonstrated for amethopterin-resistant mutants of *B. subtilis* in the following manner. It was observed that 2-methyl - 4-amino - 5-aminomethyl pyrimidine (2-CH_3-AAMP), the pyrimidine moiety of thiamine, inhibited *B. subtilis* 6051-9/A-1, a purine-requiring, amethopterin-resistant, double-mutant. When the medium was supplemented with amethopterin, however, the inhibitory effect was prevented. Because of this unexpected finding, the effect of 2-CH_3-AAMP upon amethopterin-inhibition of the wild-type parent was tested. This pyrimidine was found to block amethopterin-inhibition in this organism completely. Subsequently, studies of thiamine and cocarboxylase revealed these metabolites to be effective in preventing amethopterin-inhibition.

Because of these results, 6 analogs of the thiamine pyrimidine were obtained‡ and tested. All of these produced growth-inhibition of various strains of *B. subtilis* or *E. coli* that could be specifically blocked by thiamine pyrimidine. Results of attempts to demonstrate collateral sensitivity with these compounds are shown in Tables I and II. Three analogs of the pyrimidine moiety of thiamine, compounds 2, 4 and 5 in Table I, were the only compounds for which significant collateral sensitivity was found for both *B. subtilis* strains resistant to amethopterin, and for an *E. coli* strain resistant to certain purine antagonists. Compound 1 and 3 elicited collateral sensitivity in the *B. subtilis* strains only. Collateral sensitivity to compound 2 in two amethopterin-resistant *B. subtilis* mu-

TABLE II. Collateral Sensitivity to 2-CH_3S-AHMP* in 2 Amethopterin-Resistant *B. subtilis* Mutants.

Strain	Supplement	Optical density‡	% inhibition
6051 (parent)		.49	
	Ameth.†	.07	87
	2-CH_3S-AHMP*	.39	18
6051/A-2		.67	
	Ameth.	.68	0
	2-CH_3S-AHMP	.19	70
6051/A-3		.64	
	Ameth.	.63	3.8
	2-CH_3S-AHMP	.23	63

* 2-methylmercapto-4-amino-5-hydroxymethyl pyrimidine, 10^{-5}M.
† Amethopterin, 10^{-6}M.
‡ Optical density of culture medium. Avg four consecutive experiments. Growth 16 hr on rotary shaker at 25°C.

‡ Thiamine pyrimidine analogs were obtained through kindness of following workers: (1) 2-methylmercapto-4-amino-5-hydroxymethyl pyrimidine, from Dr. J. Gots. Prepared by Ulbricht and Price(6); (2) 2-mercapto-4-amino-5-hydroxymethyl pyrimidine, and (3) 2-ethylmercapto-4-amino-5-hydroxymethyl pyrimidine, from Dr. C. C. Stock; (4) 2-methylmercapto-4-amino-5-ethoxymethyl pyrimidine, Dr. J. Hoover; (5) 2-ethylmercapto-4-amino-5-chloromethyl pyrimidine, and (6) 2-hydroxy-4-amino-5-aminomethyl pyrimidine, from Dr. J. M. Sprague.

tants is demonstrated in Table II. One of the 6 pyrimidines, 2-hydroxy-4-amino-5-aminomethyl pyrimidine (2-HO-AAMP), differed from the others. Although growth of parent strain of *B. subtilis* was inhibited by this compound, none of the amethopterin-resistant mutants were sensitive to it, but instead showed cross-resistance, the converse of collateral sensitivity. Yet, the *E. coli* mutant resistant to 3 purine antagonists demonstrated collateral sensitivity to 2-HO-AAMP (Table I). These results indicate that one cannot predict from the effects of a pair of antimetabolites in one biological system, whether cross-resistance or collateral sensitivity will be demonstrated in a second system.

It is noteworthy that the only other compound for which *E. coli* strains resistant to purine antagonists exhibited collateral sensitivity was amethopterin, an antimetabolite of demonstrated value in acute leukemia. The results reported here support the view that, at least in some cases, collateral sensitivity to a second drug accompanying resistance to a first drug, has its basis in rather close metabolic relationships between metabolic targets of the two drugs. In this respect, the phenomenon is closely related to the inverse phenomenon of cross-resistance. The discovery of collateral sensitivity in both 6-MP and amethopterin-resistant mutant organisms for several members of a new series of pyrimidines, all analogs of the pyrimidine moiety of thiamine, permits new experiments in two directions. Work is in progress to assess the value of these pyrimidines in animal neoplasms where the effects of 6-MP and amethopterin are known, and where resistance to these agents has been established. One analog, 2-CH_3S-AHMP, in preliminary experiments has been found to retard significantly leukemia and carcinoma in mice. Elucidation of biochemical pathways relating the folic acid complex to thiamine synthesis may follow from studies of the mode of action of amethopterin and of the thiamine pyrimidine analogs in *B. subtilis* and *E. coli*. Such studies have been initiated in this laboratory.

Summary. A microbiological method for biochemical screening of compounds of potential interest in the chemotherapy of leukemia, using mutants of *B. subtilis* resistant to amethopterin and *E. coli* mutants resistant to purine-antagonists, has been described. Greater growth suppression by a test compound in the resistant strain, when contrasted to the parent strain, has been sought (collateral sensitivity). This phenomenon of collateral sensitivity has been observed for several chemical analogs of the pyrimidine moiety of thiamine. Possible significance of these findings for studies in intermediary metabolism and for cancer chemotherapy has been discussed.

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