Identification of the Antimetabolite of L-Alanosine, L-Alanosyl-5-Amino-4-Imidazolecarboxylic Acid Ribonucleotide, in Tumors and Assessment of Its Inhibition of Adenylosuccinate Synthetase

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ABSTRACT

The conjugate of L-alanosine [L-2-amino-3-(N-hydroxyN-nitrosamino)propionic acid] and 5-amino-4-imidazolecarboxylic acid ribonucleotide has been synthesized in good yield by enzymatic means, using partially purified chicken liver 5-amino-4-imidazole-N-succinocarboxamide ribonucleotide synthetase (EC 6.3.2.6). The chromatographic behavior of this molecule was characterized, as was its ability to inhibit adenylosuccinate synthetase, an enzyme long considered to be the locus of action of the drug. The Kₐ of L-alanosyl-5-amino-4-imidazolecarboxylic acid ribonucleotide versus a partially purified adenylosuccinate synthetase from the L5178Y/AR leukemia of C57BL × DBA/2 F₁ (hereafter called BD2F₁) mice was 0.228 μM, whereas the Kₐ of L-alanosine was 57.23 μM. Administration of 50 μCi of DL-[1-¹⁴C]Alanosine along with unlabeled L-alanosine (500 mg/kg) to BD2F₁, mice bearing s.c. nodules of Leukemia L5178Y/AR resulted in the accumulation in tumors of a material with properties compatible with those of L-alanosyl-5-amino-4-imidazolecarboxylic acid ribonucleotide. It coeluted with L-alanosyl-5-amino-4-imidazolecarboxylic acid ribonucleotide in the high-resolution chromatographic system used, was Bratton-Marshall positive, and inhibited adenylosuccinate synthetase strongly. In tumor nodules 2 hr after dosage, the concentration of this compound approximated 70 μM. Under the same circumstances, the intratumoral concentration of L-alanosine was found to be 440 μM. At this concentration, the antibiotic itself exerts only a marginal inhibition of leukemic adenylosuccinate synthetase. In ancillary studies, it was shown for the first time in vivo that the parenteral administration of L-alanosine reduces the specific activity of intratumoral adenylosuccinate synthetase by 70% and depresses the synthesis of DNA to an equivalent or greater extent; adenine but not hypoxanthine (both at 250 mg/kg) was able to reverse the latter inhibition. No effect on purine salvage enzymes was exerted by L-alanosine. Viewed in concert, these experiments establish that the adduct of L-alanosine with 5-amino-4-imidazolecarboxylic acid is formed by neoplastic cells in vivo and that this anabolite is most probably responsible for the inhibition of adenylosuccinate synthetase and, in turn, for the diminished synthesis of DNA seen after a therapeutic dose of L-alanosine.

INTRODUCTION

Although it has been postulated repeatedly that the active metabolite of antitumor agent L-alanosine [L-2-amino-3-(N-hydroxy, N-nitrosamino)propionic acid] is the adduct of the anti-biotic with AICOR¹ (Chart 1), attempts to demonstrate this molecule in tumors have, thus far, met with failure (10, 19). However, because its identification is central to any explanation of the mechanism of action of L-alanosine, we have undertaken additional, more comprehensive studies on the in vivo formation of L-alanosyl-AICOR. Leukemia L5178Y/AR, growing as s.c. nodules in the flanks of C57BL × DBA/2 F₁ (hereafter called BD2F₁) mice, has been used for these investigations because of its clear sensitivity to L-alanosine.

It will be demonstrated that the concentration of unaltered L-alanosine in such tumors is inadequate to explain the biochemical effects of the drug, and that, under the experimental conditions used, a compound with chromatographic and chromogenic properties of the putative adduct of L-alanosine with AICOR is, in fact, present in the neoplasms at a concentration which ought to engender total inhibition of the target enzyme, adenylosuccinate synthetase. Ancillary experiments will also be described which fortify this conclusion and quantify the extent to which L-alanosine, after its anabolism, inhibits the formation of adenylosuccinic acid in vivo, thereby restricting the concentration of adenine nucleotides required for the synthesis of DNA in the actively dividing cells of this leukemia.

MATERIALS AND METHODS

L-Alanosine (NSC 153553) was obtained from the Drug Research and Development Branch, National Cancer Institute, Bethesda, Md. DL-[1-¹⁴C]Alanosine (specific activity, 7.1 mCi/mmoll was supplied by Stanford Research Institute, Menlo Park, Calif. This radioactive alanosine was purified before use as described earlier (1). L-[4-¹⁴C]Aspartic acid (specific activity, 19.5 mCi/mmoll was obtained from Amersham/Searle Corp., Arlington Heights, III. Adenosine, hypoxanthine, ribose 1-phosphate, GTP, and IMP were purchased from Sigma Chemical Co., St. Louis, Mo. All other reagents were of the highest purity available.

Sealable polypropylene test tubes (1800 μl), products of Brinkmann Instruments, Inc., Westbury, N. Y., were used for all enzyme assays (6).

Animals. Male BD2F₁ mice on an ad libitum diet of Purina mouse chow were used during the course of these studies.

Preparation of Homogenates. BD2F₁ mice bearing 10-day-old L5178Y/AR s.c. tumors were killed by cervical dislocation;

¹ The abbreviations used are: AICOR, 5-amino-4-imidazolecarboxylic acid ribonucleotide; L-alanosyl-AICOR, L-alanosyl-5-amino-4-imidazolecarboxylic acid ribonucleotide; IMP, inosine 5'-monophosphate; DTT, dithiothreitol; TCA, trichloroacetic acid; PRPP, 5-phospho-ribose 1-pyrophosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SAICAR, 5-amino-4-imidazole-N-succinocarboxamide ribonucleotide.

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the tumors were removed, frozen, and homogenized (1:3, w/v) in 0.1 M Tris-HCl buffer (pH 7.6), with 0.5 mM EDTA and 1 mM DTT. For measurement of the concentrations of L-alanosine and L-alanosyl-AICOR, the tumors were homogenized in 1 N acetic acid (1:3, w/v). For measurement of the purine salvage enzymes, the tumors were homogenized in 0.1 M Tris-HCl buffer (pH 7.0), containing 10 mM DTT. Homogenates were centrifuged at 12,000 × g for 3 min and the supernatant was used as enzyme source.

**Protein Estimation.** Protein was estimated using the Bio-Rad kit for protein determination (2). Ten-μl aliquots of a suitably diluted supernatant solution were added to 1.0 ml of dye solution, prepared, and diluted according to the manufacturer’s directions. The absorbance at 595 nm was then measured within 5 to 60 min. Aliquots of a solution of bovine albumin, Fr 5, (1 mg/ml), were used as standards.

**Concentration of L-Alanosine in L5178Y/AR Tumors.** L5178Y/AR s.c. tumors were transplanted in BD2F1 mice. Ten days later, the animals were fasted overnight and treated with alanosine (DL-[1-14C]alanosine, 10 μCi/mouse, along with cold L-alanosine, 500 mg/kg). At appropriate intervals, groups of 6 animals were sacrificed, and the tumors were removed and homogenized in 1 N acetic acid. The homogenates were centrifuged for 12 min at 12,000 × g. The concentration of L-alanosine in the tumors was calculated within 5 to 60 min. Aliquots of a solution of bovine albumin, Fr 5, (1 mg/ml), were used as standards.

**Effects of L-Alanosine on DNA Synthesis.** One million cells were administered i.p. to 6 groups of 6 mice each. The first group received L-alanosine (500 mg/kg) i.p., and the second and third groups received adenosine and hypoxanthine (250 mg/kg), respectively. The last group received a 0.25 ml of 0.9% sodium chloride solution. After 4 hr, all animals were given 5 μCi of [2-14C]thymidine. One hr later, the recipients were sacrificed, and tumors were removed, frozen, and later homogenized in 5% TCA. After centrifugation for 3 min at 12,000 × g, the pellet was washed 3 times with 1% TCA. Aliquots of 1 ml of 10% TCA were then added to the pellets, mixed well, and heated at 95° for 1 hr. A suitable aliquot of the supernatant was then used for estimation of radioactivity and measurement of DNA (11).

**Hypoxanthine and Adenine Phosphoribosyltransferase.** The following reagents were admixed in Eppendorf tubes: 10 μl of [8-14C]hypoxanthine (6 nmol, 332 nCi), 10 μl of PRPP and MgCl2 (60 and 180 nmol, respectively, in 0.2 mM Tris buffer, pH 8.0), and 10 μl of tumor extract to initiate the reaction. In control vessels, PRPP was replaced by 10 μl of 0.2 mM Tris buffer, pH 8.0. Incubation was carried out at 37° for 30 min; thereafter the reaction was stopped by adding 20 μl of 2 N HCl. After a 3-min centrifugation at 12,000 × g, 5 μl of the supernatant were spotted on Whatman 3M paper and overspotted with 5 μl of a mixture of IMP and hypoxanthine (0.01 μM each). Ascending chromatograms were developed using 1 mM ammonium acetate (pH 7.0); ethanol (30:70, v/v), as solvent for 16 hr. The IMP spots were then excised and their radioactivity was measured. For calculation of enzyme activity, the radioactivity in the vessels lacking PRPP was subtracted from that measured in the vessels receiving this substrate.

In the case of adenine phosphoribosyl transferase, [8-14C]hypoxanthine was replaced by 10 μl of [8-14C]adenine (6.0 nmol, 314 nCi), and the chromatograms were overspotted with adenine and AMP, developed, and processed as described above.

**Adenosine Kinase.** In Eppendorf test tubes were admixed 5 μl of [8-14C]adenosine (7 nmol, 455 nCi), 5 μl of 0.05 mM HEPES buffer (pH 6.8), 5 μl of ATP and MgCl2 (20 and 6 nmol, respectively, in 0.05 mM HEPES buffer, pH 6.8), and 5 μl of enzyme extract. Control vessels received HEPES buffer instead of ATP and MgCl2. The reactants were incubated for 15 min at 37°, at which point 20 μl of 2 N HCl containing 0.01 mM AMP were added to arrest the reaction. Five-μl aliquots of the resultant reaction mixtures were then spotted on Whatman 3M paper. Ascending chromatography was performed using isopropyl alcohol:EDTA (saturated solution):toluene:14 mM NH4OH (320:44:40:4, v/v) as solvent for 16 hr (12). The UV-absorbing spots of AMP at the origin were excised, and their radioactivity was measured by scintillation spectrometry.

**Inosine Phosphorylase.** In Eppendorf tubes, the following were admixed: 10 μl of [8-14C]hypoxanthine (8 nmol, 442 nCi); 10 μl of ribose 1-phosphate (300 nmol in 0.1 M Tris buffer, pH 7.0); and 10 μl of enzyme extract. Control vessels received 10 μl of 0.1 M Tris buffer, pH 7.0, in place of ribose 1-phosphate. All vessels were incubated at 37° for 15 min, and the reaction was stopped by adding 20 μl of 2 N HCl. For the separation of hypoxanthine and inosine, 5-μl aliquots of these reaction mixtures were spotted on Polygram CEL 300 polyethyleneimine plates and overspotted with 5 μl of a mixture of inosine and hypoxanthine (0.01 μM each). The plates were developed in distilled water for 2 hr (14), and spots of inosine were excised for scintillation spectrometry.
Adenosine Phosphorylase. The following were admixed: 5 \(\mu\)l of \([8-\text{C}^14]\)adenosine (7 nmols, 455 nCi); 5 \(\mu\)l of 0.2 M HEPES buffer, pH 7.0; 5 \(\mu\)l of 0.05 M potassium phosphate solution, pH 7.0, and 5 \(\mu\)l of enzyme extract. Control vessels contained 5 \(\mu\)l of HEPES buffer in place of phosphate. The vessels were incubated at 37° for 15 min, and the reaction was stopped by adding 20 \(\mu\)l of 2 M HCl; 5 \(\mu\)l aliquots of these reaction mixtures were spotted on Whatmann 3M chromatography paper and overspotted with 5 \(\mu\)l of a mixture of adenine and adenosine (0.01 \(\mu\)m each). The chromatograms were developed in an ascending manner using 1 M ammonium acetate (pH 7.0); alcohol (30:70, v/v) as solvent for 16 hr. The UV spots of adenosine were excised, and their radioactivity was measured by scintillation spectrometry.

Adenylosuccinate Synthetase Activity. Adenylosuccinate synthetase activity was measured according to a published procedure (18). Briefly, in a total volume of 20 \(\mu\)l were admixed: 5 \(\mu\)l of L-[4-\text{C}]aspartic acid (6.37 nmol, 125 nCi); 5 \(\mu\)l of GTP-MgCl\(_2\) (150 nmol each); 5 \(\mu\)l of IMP (150 nmol); and, to initiate the reaction, 5 \(\mu\)l of tumor extract. Controls were constituted by replacing IMP with 5 \(\mu\)l (200 nmol) of Tris-HCl buffer, pH 8.0. Incubation was carried out for 30 min at 37°. The reaction was stopped by heating the vessels at 95° for 2 min. Unreacted L-aspartic acid was then decarboxylated by the addition of 50 \(\mu\)l of a decarboxylation reagent (17) consisting of 0.005 M \(\alpha\)-ketoglutarate and 0.03 M zinc sulfate in 0.66 M sodium acetate buffer, pH 5.0; to this solution, 40 IU/ml of L-glutamate-oxaloacetate transaminase were added at the time of use. After 3 hr at 37°, the radioactive residues in the vessels were counted by scintillation spectrometry in a Beckman LS-230 scintillation spectrometer.

Inhibition of Adenylosuccinate Synthetase from L5178Y/AR by L-Alanosine in Vivo. One million L5178Y/AR cells were implanted s.c. in the flanks of BALB/c mice. Ten days later, the animals were fasted overnight and divided into two groups. The first group received an i.p. dose of L-alanosine (500 mg/kg), and the second group received an equal volume of 0.9% sodium chloride solution. At the designated time intervals, 5 mice from each group were sacrificed by cervical dislocation, and tumors were dissected and frozen on solid CO\(_2\). Within 72 hr, the tumors were homogenized, and adenylosuccinate synthetase activity was measured as described earlier.

Purification of Adenylosuccinate Synthetase from L5178Y/AR Tumors. Mice bearing s.c. nodules of leukemia L5178Y/AR were sacrificed, and tumors were removed and homogenized in 0.02 M potassium phosphate buffer containing 0.5 mM EDTA and 2.0 mM DTT (1:3, w/v). The homogenate was centrifuged at 20,000 x g for 30 min. The clear supernatant was then placed in a water bath and heated at 50° for 5 min. Thereafter, the solution was immediately cooled in an ice bath. The proteins thus precipitated were removed by centrifugation at 20,000 x g for 20 min. The resulting supernatant was dialyzed for 5 hr against 0.001 M potassium phosphate buffer containing 0.5 mM EDTA and 2 mM DTT and loaded onto a column of hydroxylapatite (2.3 x 40 cm) equilibrated with 0.001 M potassium phosphate buffer containing 0.5 mM EDTA and 2.0 mM DTT. The column was first developed with 200 ml of equilibrating buffer, followed by a linear gradient of potassium phosphate buffer from 0.001 to 0.25 M, pH 7.2 (250 ml each), containing 0.5 mM EDTA and 2 mM DTT. Fractions of 10 ml each were collected. Adenylosuccinate synthetase eluted from this column at 0.115 M potassium phosphate buffer. The active fractions were pooled and concentrated by dialysis against Carbowax. This enzyme preparation was 15-fold purified over the crude and exhibited a specific activity of 137.47 units/mg protein (1 unit of enzyme represents the amount of protein responsible for the production of 1 nmol of adenylosuccinic acid per hr). The heating step was especially useful for removing adenylosuccinate lyase, which uses adenylosuccinic acid as one of its substrates. We also tested this preparation for "IMP phosphatase" activity. When 3.44 nmol (100 nCi) of IMP were incubated with 5 \(\mu\)l of purified enzyme at 37° for 30 min less than 0.034 nmol of IMP were hydrolyzed; this corresponds to less than 1.0% decomposition.

Kinetics of Inhibition of Adenylosuccinate Synthetase In Vitro by L-Alanosine. For this study, partially purified adenylosuccinate synthetase was used. The reaction mixture in a total volume of 25 \(\mu\)l contained: 5 \(\mu\)l of L-[4-\text{C}]aspartic acid (0.78 to 12.5 mm); 5 \(\mu\)l of GTP-MgCl\(_2\) (0.1 to 10 mm each) in 0.05 M Tris-HCl, pH 8.0; 5 \(\mu\)l of IMP (0.1 to 10 mm) in 0.05 M Tris-HCl, pH 8.0; or 5 \(\mu\)l of buffer and 5 \(\mu\)l of L-alanosine (15 to 60 mm) in 0.05 M Tris-HCl, pH 8.0; or 5 \(\mu\)l of partially purified adenylosuccinate synthetase from L5178Y/AR (3.45 units). When IMP was the variable substrate (0.1 to 10 mm), the concentration of GTP and L-aspartic acid were kept constant at 5.0 and 10.0 mm, respectively. Similarly, the concentrations of IMP and L-aspartic acid were fixed at 5.0 and 10.0 mm, respectively, when GTP was the variable substrate (0.1 to 10.0 mm). The reactions were mixed by a brief centrifugation at 12,000 x g and incubated at 37° for 30 min. The reaction was terminated by heating at 95° for 2 min, and incubation mixtures were processed as detailed earlier. The kinetic constants were derived from Lineweaver-Burk plots.

SAICAR Synthetase. SAICAR synthetase was measured as follows. In a total volume of 25 \(\mu\)l were admixed: 10 \(\mu\)l of L-[4-\text{C}]aspartic acid (12.74 nmol, 250 nCi); 5 \(\mu\)l of ATP-MgCl\(_2\) (75 nmol each); 5 \(\mu\)l of AICOR (30 nmol in 0.1 M KHCO\(_3\)) or 5 \(\mu\)l of 0.05 M Tris-HCl buffer, pH 8.0; and, to initiate the reactions, 5 \(\mu\)l of enzyme extract. The reactants were mixed by a brief centrifugation of 12,000 x g and incubated at 37° for 30 min; thereafter, the reaction was stopped by heating at 95° for 2 min. The vessels were opened, 50 \(\mu\)l of decarboxylation reagent was added, and the assemblies were incubated at 37° for 3 hr to dissipate residual L-[4-\text{C}^14]aspartic acid. Radioactivity remaining in the vessels was then counted by scintillation spectrometry. For calculations, radioactivity in vessels lacking AICOR was subtracted from radioactivity in vessels receiving this additive, and the difference was taken as a measure of enzyme activity.

Purification of SAICAR Synthetase from Chicken Liver. A partially purified preparation of this enzyme was obtained by a modification of the method of Buchanan et al. (4). Thus, 10 g of an acetone powder prepared from fresh chicken liver were extracted at 2° by stirring for 30 min with 100 ml of 0.05 M potassium phosphate buffer at pH 7.3. The crude extract was treated with pancreatic RNase, 0.2 mg of a crystalline preparation being added per ml of enzyme solution. The mixture was allowed to come to 37° over a period of 10 to 15 min in a constant temperature bath. The small amount of precipitate brought about by this procedure was centrifuged at 4°. Sufficient solid ammonium sulfate was then added to the supernatant solution to bring the salt concentration to 55% of satura-
tion. The precipitate, which contained all the activity, was collected by centrifugation, dissolved in 100 ml of 0.05 M potassium phosphate buffer, (pH 7.3), and refractionated with ammonium sulfate. The protein precipitating between 25 and 45% of saturation was collected by centrifugation and dissolved in 20 ml of 0.02 M phosphate buffer (pH 7.3). This enzyme solution was dialyzed against 2 changes of 4 liters each of 0.01 M phosphate buffer (pH 7.3) containing 1 mM DTT. The dialyzed material was diluted 2-fold with cold water and loaded onto a column (2.5 x 40 cm) of hydroxylapatite. The column was developed with a linear gradient of 0.05 to 0.35 M phosphate buffer (pH 7.0) containing 1 mM DTT. The enzyme eluted at 0.18 M phosphate. Active fractions were pooled and frozen. The enzyme was purified 35.0-fold and exhibited a specific activity of 955.0 nmol per mg protein per hr.

The synthesis of AICOR was carried out as described earlier (18).

Preparation and Purification of L-Alanosyl-AICOR. L-Alanosyl-AICOR was prepared by condensing L-alanosine with AICOR via the action of SAICAR synthetase from chicken liver. The following were dissolved in 200 μl of water: 6 mg ATP; 2 mg MgCl₂; 8.0 mg KHCO₃, 3.0 mg DL-[1-¹⁴C]alanosine (specific activity, 7.4 mCi/mmol); and 4.0 mg AICOR. To this mixture were added 2.8 ml of purified chicken liver enzyme, and the reactants were incubated at 37° for 6 hr. The incubation mixture was then deproteinized with 0.1 N HCl and centrifuged at 12,000 x g for 12 min. A portion of this supernatant (0.8 ml) was then loaded on a 0.8- x 15-cm Hamilton HA-X4 column equilibrated with 0.015 M lithium citrate buffer, pH 2.65 (20), and operated at a flow rate of 0.67 ml/min. The column was developed with the equilibrating buffer for 90 min at 37°, followed by 0.0375, 0.075, 0.15, and 0.225 M lithium citrate buffers (pH 2.72), applied for periods of 60, 60, 75, and 60 min each (20). L-Alanosyl-AICOR eluted from this column as a single symmetrical peak at ~260 min. For desalting, the peak fractions from this chromatography were collected, lyophilized, reconstituted in 1 ml, and loaded on an 0.8- x 15-cm column of Aminex A-14 resin in the bicarbonate form. The column was developed with a linear gradient of 0 to 1.0 M ammonium bicarbonate. The L-alanosyl-AICOR eluted from this column at 240 min; it was collected, frozen, lyophilized repeatedly, and stored at −70°. The yield of L-alanosyl-AICOR from this procedure was ~5%.

This preparation of L-Alanosyl-AICOR gave a single spot on paper electrophoresis (at 25 V/cm in 50 mM sodium phosphate buffer, pH 7.2) and thin-layer chromatography, using Baker Flex Silica Gel IB-F precoated plates with n-butyl alcohol:acetic acid:water (5:3:2) as solvent. The compound was Bratton-Marshall positive (3, 16) and a very strong inhibitor of adenylosuccinate synthetase. Hence, for preparing L-alanosyl-AICOR from tumors to test on adenylosuccinate synthetase, a 0 to 1.0 M ammonium bicarbonate gradient was utilized. Thus, tumors were homogenized in 1 N acetic acid, the pH was adjusted back to 7.0 with HCl, the extracts were clarified by centrifugation, and a portion of the supernatant (0.8 ml) was loaded on a Hamilton HA-X4 column (0.8 x 15.0 cm; HCO₃⁻ form) preequilibrated with distilled water. The column was developed with a 0 to 0.1 M ammonium bicarbonate gradient (100 ml each). L-Alanosine and L-alanosyl-AICOR eluted in this system at 75 and 240 min, respectively. Fractions of 3.35 ml were collected. A 500-μl aliquot from each fraction was then taken for the measurement of radioactivity, and the remainder was lyophilized. The products of lyophilization were reconstituted in 1.0 ml of water and relyophilized to remove traces of ammonium bicarbonate. All fractions were then separately reconstituted in 25 μl of water. To test these fractions for the inhibition of adenylosuccinate synthetase, the following were admixed in a total volume of 25 μl: 5 μl of L-[4-¹⁴C]aspartic acid (6.37 nmol, 125 nCi); 5 μl of GTP-MgCl₂ (125 nmol each); 5 μl of IMP (125

to pH 2 with HCl, there was a 30% loss in absorbance even though it retained the same λₘₐₓ. Based on the specific activity of the radioactive alanosine used to synthesize it, this compound had a molar extinction coefficient of 5.75 x 10⁴.

Identification and Concentration of L-Alanosyl-AICOR in L5178Y/AR Tumors. Mice bearing Leukemia L5178Y/AR were treated with DL-[1-¹⁴C]alanosine, 50 μCi/mouse, along with cold L-alanosine, 500 mg/kg. After 2, 4, and 8 hr the animals were sacrificed, and tumors were removed, frozen, homogenized in 1 N acetic acid, and centrifuged for 12 min at 12,000 x g. A portion of the resulting supernatant (0.8 ml) was loaded on a 0.8- x 15.0 cm of column of Hamilton HA-X4 resin, and elution was performed as described above. The concentration of L-alanosyl-AICOR was calculated on the basis of the specific radioactivity of the injection material.

Lithium citrate, used for eluting the above column, inhibits adenylosuccinate synthetase. Hence, for preparing L-alanosyl-AICOR from tumors to test on adenylosuccinate synthetase, a 0 to 1.0 M ammonium bicarbonate gradient was utilized. Thus, tumors were homogenized in 1 N acetic acid, the pH was adjusted back to 7.0 with HCl, the extracts were clarified by centrifugation, and a portion of the supernatant (0.8 ml) was loaded on a Hamilton HA-X4 column (0.8 x 15.0 cm; HCO₃⁻ form) preequilibrated with distilled water. The column was developed with a 0 to 0.1 M ammonium bicarbonate gradient (100 ml each). L-Alanosine and L-alanosyl-AICOR eluted in this system at 75 and 240 min, respectively. Fractions of 3.35 ml were collected. A 500-μl aliquot from each fraction was then taken for the measurement of radioactivity, and the remainder was lyophilized. The products of lyophilization were reconstituted in 1.0 ml of water and relyophilized to remove traces of ammonium bicarbonate. All fractions were then separately reconstituted in 25 μl of water. To test these fractions for the inhibition of adenylosuccinate synthetase, the following were admixed in a total volume of 25 μl: 5 μl of L-[4-¹⁴C]aspartic acid (6.37 nmol, 125 nCi); 5 μl of GTP-MgCl₂ (125 nmol each); 5 μl of IMP (125

2 The Bratton-Marshall reaction was performed as follows: To 10 μl of sample on ice were added 20 μl of 10 N H₂SO₄, and 10 μl of 0.1% sodium nitrite. After 1 min, 10 μl of 0.5% ammonium sulfate were added to remove excess sodium nitrite. Three min later, 10 μl of 1.0% naphthyl ethylenediamine HCl were added. L-Alanosyl-AICOR yielded a purplish color which was stable if samples were maintained in the cold. For determining the sensitivity of this procedure, various concentrations of L-alanosyl-AICOR were used; it was observed that 20 nmol of L-alanosyl-AICOR was the lower limit of detection under these experimental conditions.
nmol) or 5 μl of 0.05 M Tris buffer, pH 8.0); 5-μl aliquot from each reconstituted fraction; and, to initiate the reaction, 5 μl (3.45 units) of adenylsuccinate synthetase partially purified from Leukemia L5178Y/AR. The reaction was carried out exactly as described earlier. Inasmuch as the concentration of L-alanosyl-AICOR was low, the remaining 20-μl aliquot of every 4 consecutive fractions were pooled, relyophilized, reconstituted in 10 μl of water, and subjected to the Bradford-Marshall reaction.

Kinetics of Inhibition of Adenylosuccinate Synthetase in Vitro by L-Alanosyl-AICOR. Kinetic studies were performed by mixing the following reagents in sequence at 4 °C in a total volume of 25 μl: 5 μl of L-[4-14C]aspartic acid (0.78 to 12.5 nmol); 5 μl of GTP-MgCl₂ (0.1 to 10 μM each) in 0.05 M Tris-HCl (pH 8.0); 5 μl of IMP (0.1 to 10 mM) in 0.05 M Tris-HCl (pH 8.0) or 5 μl of buffer alone; 5 μl of L-alanosyl-AICOR (2.4 to 19.5 μM) in 0.05 M Tris-HCl (pH 8.0) or 5 μl of buffer alone; and 5 μl of partially purified adenylosuccinate synthetase from L5178Y/AR (3.45 units). The reaction was started by brief centrifugation at 12,000 x g for 20 sec, and the vessels were incubated at 37 °C for 30 min. The reaction was terminated by heating at 95 °C for 2 min, and the product was analyzed as described earlier. When the concentration of IMP was varied (0.1 to 10.0 mM), L-aspartic acid and GTP were fixed at 10.0 and 5.0 mM, respectively. Similarly, at variable concentrations of GTP (0.1 to 10.0 μM), the concentrations of L-aspartic acid and IMP were fixed at 10.0 and 5.0 mM, respectively. The reaction was carried out as described earlier, and the kinetic constants were derived from Lineweaver-Burk plots.

RESULTS

Inhibition of DNA Synthesis by L-Alanosine. Previous workers using cells in culture have established that L-alanosine inhibits the incorporation of [14C]formate into adenine but not into guanine (7). In addition, Novikoff rat hepatoma cells treated with L-alanosine accumulate IMP (8). These findings strongly suggest that adenylosuccinate synthetase is the primary site of action of L-alanosine. Inasmuch as the concentration of ATP is depressed by L-alanosine both in normal liver and in tumors (1), it would be expected that dATP and DNA synthesis would be affected in turn. As Table 1 documents, a single dose of L-alanosine (500 mg/kg) given to mice bearing s.c. nodules of Leukemia L5178Y/AR inhibited the synthesis of DNA to a prominent degree. To extend these studies, we next determined whether the inhibition of L-alanosine on DNA synthesis could be reversed by adenine and hypoxanthine; the former base can be converted directly to AMP via the action of adenine phosphoribosyltransferase and bypass the adenylosuccinate synthetase step, while hypoxanthine cannot. As Table 1 shows, when hypoxanthine (250 mg/kg) was given along with L-alanosine, no reversal of the inhibition of DNA synthesis was observed; whereas the same dose of adenine almost completely reversed the inhibition of DNA synthesis produced by administration of the drug.

The Influence of L-Alanosine on the Enzymes of Purine Salvage. The foregoing studies suggested that the enzymes responsible for the salvage of adenine might modulate the therapeutic activity of L-alanosine by catalyzing the reutilization of this base, which as documented is an efficacious antidote against the toxicity of the antibiotic. In addition, the possibility was entertained that the state of adenine deprivation produced by the administration of L-alanosine might induce the enzymes of adenine salvage; such induction would be of obvious therapeutic concern. However, as Table 2 documents, no differences in these systems were discerned either basally or after provocation with L-alanosine given at a dose of 500 mg/kg.

Inhibition of Adenylosuccinate Synthetase by L-Alanosine In Vitro and in Vivo. Clark and Rudolph (5) reported that L-alanosine itself is a rather weak inhibitor of Novikoff ascites tumor adenylosuccinate synthetase. Hurlbert et al. (10) suggested that the antitumor effect of L-alanosine was exerted by an anabolite of the drug and not by the drug itself. Our first step to confirm this contention sought to compare the magnitude of inhibition of adenylosuccinate synthetase produced by L-alanosine in vitro with that exerted by the drug in vivo.

Adenylosuccinate synthetase partially purified from Leukemia L5178Y/AR was used for kinetic studies in vitro. Inasmuch as L-alanosine is an analog of L-aspartic acid, we first studied the inhibition of this enzyme by L-alanosine using L-aspartic acid as the variable substrate; the inhibition observed was noncompetitive and weak, with a Kᵢ of 57.23 μM (Chart 3). In the interest of completeness, we further studied the inhibition of adenylosuccinate synthetase by L-alanosine using GTP and IMP as variable substrates. Inhibition was noncompetitive against these substrates also, with a Kᵢ of 30.42 μM for GTP and 37.10 μM for IMP (Table 3).

Table 4 presents the effect of L-alanosine on adenylosuccinate synthetase activity of Leukemia L5178Y/AR. The reaction was carried out exactly as described earlier. Inasmuch as L-alanosine is analog of L-aspartic acid, we first studied the inhibition of this enzyme by L-alanosine using L-aspartic acid as the variable substrate; the inhibition observed was noncompetitive and weak, with a Kᵢ of 57.23 μM (Chart 3). In the interest of completeness, we further studied the inhibition of adenylosuccinate synthetase by L-alanosine using GTP and IMP as variable substrates. Inhibition was noncompetitive against these substrates also, with a Kᵢ of 30.42 μM for GTP and 37.10 μM for IMP (Table 3).

Table 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>[2-14C]Thymidine (nCi/mg DNA)</th>
<th>% of inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.9% NaCl solution</td>
<td>4.63 ± 0.29</td>
<td>0</td>
</tr>
<tr>
<td>L-Alanosine</td>
<td>0.69 ± 0.19</td>
<td>85.1</td>
</tr>
<tr>
<td>L-Alanosine + adenine</td>
<td>4.10 ± 0.10</td>
<td>11.45</td>
</tr>
<tr>
<td>L-Alanosine + hypoxanthine</td>
<td>0.71 ± 0.15</td>
<td>84.67</td>
</tr>
<tr>
<td>Hypoxanthine</td>
<td>4.72 ± 0.88</td>
<td>0</td>
</tr>
<tr>
<td>Adenine</td>
<td>4.49 ± 0.41</td>
<td>3.30</td>
</tr>
</tbody>
</table>

* Mean ± S.D.

a Significantly different from the control group (p < 0.002).

Table 2

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Specific activity (nmol/mg protein/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenine phosphoribosyl-</td>
<td>25.83 ± 2.09</td>
</tr>
<tr>
<td>transferase</td>
<td></td>
</tr>
<tr>
<td>Hypoxanthine phosphoribosyl-</td>
<td>30.98 ± 2.56</td>
</tr>
<tr>
<td>transferase</td>
<td></td>
</tr>
<tr>
<td>Adenosine kinase</td>
<td>27.63 ± 3.84</td>
</tr>
<tr>
<td>Adenosine phosphorylase</td>
<td>11.33 ± 2.48</td>
</tr>
<tr>
<td>Inosine phosphorylase</td>
<td>90.18 ± 10.17</td>
</tr>
</tbody>
</table>

* Mean ± S.D.
Mechanism of Action of L-Alanosine

hr the antibiotic inhibited tumoral adenylosuccinate synthetase by about 70%. Thereafter, gradual restitution was observed. Greater than 90% of the inhibition observed was reversible by dialysis.

**Concentration of L-Alanosine in L5178Y/AR Tumors.** Since L-Alanosine itself can inhibit adenylosuccinate synthetase, it became important to determine whether the nodules of Leukemia L5178Y/AR used in the studies described earlier contained the antibiotic *in vivo* at a concentration commensurate with the kinetics of inhibition measured *in vitro*. Hence, as a next step, we measured the concentration of L-alanosine in these tumors. It was found that the concentration of unaltered L-alanosine fell to 440 μM within 2 hr (Table 5) after the administration of the drug. By 4 and 8 hr, the concentration of the drug decreased further despite the fact that the inhibition of adenylosuccinate synthetase had been found to persist at ~70% over this time span. These concentrations of L-alanosine were incapable of exerting the magnitude of enzyme inhibition observed, a finding which ruled out the possibility that L-alanosine itself could be functioning *in vivo* as the proximate inhibitor of adenylosuccinate synthetase.

**Condensation of L-Alanosine with AICOR.** Since the concentration of L-alanosine in tumors was inadequate to explain the strong inhibition of adenylosuccinate synthetase produced by it, support was sought for the hypothesis that an anabolite of the drug, possibly L-alanosyl-AICOR, was responsible for the action of the antibiotic *in vivo*.

Preparatory to these studies, "authentic" L-alanosyl-AICOR was prepared from L-alanosine and AICOR by the catalytic action of a preparation of SAICAR synthetase partially purified from avian liver. This compound was homogeneous on electrophoresis and thin-layer chromatography, a strong inhibitor of adenylosuccinate synthetase, and Bratton-Marshall positive. It eluted as a symmetrical peak at 260 min from a high-resolution column of Hamilton HA-X4 resin developed as described previously (20). Thus, although strict chemical identity was established, the material thus isolated was utilized as a provisional but plausible standard in the work to be described.

**Inhibition of Adenylosuccinate Synthetase in Vitro by L-Alanosyl-AICOR.** In order to determine the inhibitory potency of L-alanosyl-AICOR, we next studied in detail the effect of this antimetabolite on adenylosuccinate synthetase, partially purified from Leukemia L5178Y/AR. When L-aspartic acid and GTP were used as variable substrates, L-alanosyl-AICOR inhibited adenylosuccinate synthetase in a noncompetitive manner with apparent Kᵈ’s of 11.6 and 8.8 μM, respectively (Table 5).

**Table 5**

<table>
<thead>
<tr>
<th>Time after L-alanosine administration (hr)</th>
<th>Concentration of L-alanosine (μM)</th>
<th>Concentration of L-alanosyl-AICOR (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>442 ± 39.2 *</td>
<td>70.2 ± 9.6</td>
</tr>
<tr>
<td>4</td>
<td>330 ± 24.8</td>
<td>53.1 ± 12.2</td>
</tr>
<tr>
<td>8</td>
<td>170 ± 15.2</td>
<td>20.3 ± 3.6</td>
</tr>
</tbody>
</table>

* Mean ± S.D.

**Table 4**

<table>
<thead>
<tr>
<th>Effect of L-alanosine on adenylosuccinate synthetase in <em>vivo</em> in L5178Y/AR tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>BD2F₂ mice bearing 10-day-old s.c. L5178Y/AR tumors were given a single i.p. dose of L-alanosine (500 mg/kg). Animals in control groups received the same volume of 0.9% NaCl solution. At designated time periods, five L-alanosine-treated and five 0.9% NaCl solution-treated animals were sacrificed, and the tumors were removed, frozen, and homogenized. Adenylosuccinate synthetase activity was then measured as described in &quot;Materials and Methods.&quot;</td>
</tr>
<tr>
<td>Time (hr)</td>
</tr>
<tr>
<td>0.5</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>4</td>
</tr>
<tr>
<td>8</td>
</tr>
<tr>
<td>24</td>
</tr>
<tr>
<td>48</td>
</tr>
</tbody>
</table>

* Mean ± S.D.; p < 0.001.

rate synthetase *in vivo*. When a therapeutic dose of the drug (500 mg/kg) was given to mice bearing nodules of Leukemia L5178Y/AR and the inhibition of adenylosuccinate synthetase was followed over time, it was observed that from 30 min to 8
However, as shown in Chart 4, the anabolite was a formally competitive inhibitor versus IMP with an apparent $K_i$ of 0.228 µM.

**Presence and Concentration of L-Alanosyl-AICOR in L5178Y/AR Tumors.** Inasmuch as it was possible to condense L-alanosine with AICOR *in vitro* and the resulting antimetabolite, L-alanosyl-AICOR, was a very potent inhibitor of adenylosuccinate synthetase, a search was mounted for L-alanosyl-AICOR in tumors. Mice bearing L5178Y/AR nodules were given 10 µCi of DL-[1-14C]alanosine along with L-alanosine, 500 mg/kg, and the tumors were removed and analyzed for L-alanosyl-AICOR. In another approach, radioactive alanosine was given to mice along with 5-amino-4-imidazolecarboxamide riboside as a precursor for AICOR. L-Alanosine-sensitive P388 and neuroblastoma cells in tissue culture were also carried through similar experimental approaches. Nevertheless, all of our initial efforts to identify this anabolite *in vivo* met with failure (19). However, when a 5-fold greater dose of radioactive DL-[1-14C]alanosine (50 µCi/animal) was given, along with a therapeutic dose of L-alanosine (500 mg/kg), to mice bearing nodules of Leukemia L5178Y/AR, and the chromatography was performed on a HA-X4 column with lithium citrate buffers, a prominent radioactive peak, coeluting with L-alanosyl-AICOR, was detectable in the tumors of the recipients at concentrations of 70, 53, and 20 µM at 2, 4, and 8 hr, respectively (Table 5). As described in "Materials and Methods" for preparing L-alanosyl-AICOR from tumors to test on adenylosuccinate synthetase, a 0 to 1.0 M ammonium bicarbonate gradient was utilized, and all chromatographic fractions from these studies were tested for inhibition of partially purified adenylosuccinate synthetase from L5178Y/AR and Bratton-Marshall positivity. Fractions corresponding to the alanosine peak had no effect on adenylosuccinate synthetase (Chart 5); however, those corresponding to the peak coeluting with alanosyl-AICOR strongly inhibited the partially purified preparation of adenylosuccinate synthetase (Chart 5). Only the fractions corresponding to this peak were served to be Bratton-Marshall positive (Chart 5). The presence of a radioactive peak, coeluting with L-alanosyl-AICOR, and its remarkable capability to inhibit adenylosuccinate synthetase and Bratton-Marshall positivity (Chart 5) strongly suggest that the compound is L-alanosyl-AICOR.

**DISCUSSION**

In its capacity as a structural and functional analog of the dicarboxylic amino acids, L-alanosine is metabolized extensively by several of the enzymes which ordinarily operate on L-aspartic acid (1, 13, 15). Therapeutically speaking, the most important such enzyme is thought to be SAICAR synthetase because it uses L-alanosine as a fraudulent substrate and generates L-alanosyl-AICOR, an antimitabolite known to inhibit adenylosuccinate synthetase with notable potency (Refs. 9, 10, and 19; Chart 4).

Up to the present, attempts to determine whether this chain of events was operative *in vivo* have met with failure (19). However, using a number of novel approaches, we have now succeeded in demonstrating that administration of L-alanosine does inhibit adenylosuccinate acid synthetase in s.c. tumor nodules and that, as a consequence of this inhibition, DNA synthesis is reduced strongly. It is especially relevant in this regard that adenine, but not hypoxanthine, reverses the depression of nucleic acid biosynthesis engendered by parenteral doses of L-alanosine. Inasmuch as hypoxanthine alone of this pair of purines must be metabolized via adenylosuccinate syn-
the effects of L-alanosine provides a cogent indication of the locus of action of the drug in vivo.

Experiments recounted in the present paper also establish that L-alanosine is present in tumors at concentrations inadequate to explain the protracted inhibition if adenylosuccinate synthetase measured (Table 4). Conversely, a strongly acidic anabolite of L-alanosine is detectable in tumors if appropriate doses of radiolabeled drug are administered; this anabolite, extracted from tumor nodules, coelutes with L-alanosyl-AICOR on high-resolution chromatography, is Bratton-Marshall positive, and inhibits adenylosuccinic acid synthetase from L5178Y/AR tumor cells with an apparent Kᵦ of 0.228 µM. On the basis of these properties, it is concluded to be L-alanosyl-AICOR. Moreover, because the concentration of this anabolite in tumors approximates 70 µM, a level ~30 times greater than its apparent Kᵦ, it is a very plausible candidate for the role of proximate antimetabolite of L-alanosine in vivo.

At present L-alanosine is undergoing Phase I and II trials in a panel of human cancers. In view of the pivotal roles that SAICAR synthetase and L-alanosyl-AICOR play in determining the efficacy of this antibiotic versus experimental tumors, efforts will be mounted to measure them in specimens of human neoplasms. It is hoped that such measurements will have predictive value. Additionally, in view of the potency of L-alanosyl-AICOR, synthesis of this anabolite will be attempted in anticipation of its use as a novel oncolytic drug in its own right.

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REFERENCES


Identification of the Antimetabolite of l-Alanosine, l-Alanosyl-5-Amino-4-Imidazolecarboxylic Acid Ribonucleotide, in Tumors and Assessment of Its Inhibition of Adenylosuccinate Synthetase

Anil K. Tyagi and David A. Cooney


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