

# The Aromatic-L-Amino Acid Decarboxylase Inhibitor Carbidopa Is Selectively Cytotoxic to Human Pulmonary Carcinoid and Small Cell Lung Carcinoma Cells<sup>1</sup>

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## ABSTRACT

The carcinoid tumor is an uncommon neuroendocrine neoplasm the hallmark of which is excessive serotonin production. In studying kinetics of tryptophan hydroxylase and aromatic-L-amino acid decarboxylase (AAAD) in human carcinoid hepatic metastases and adjacent normal liver (J. A. Gilbert *et al.*, *Biochem. Pharmacol.*, 50: 845–850, 1995), we identified one significant difference: the  $V_{\max}$  of carcinoid AAAD was 50-fold higher than that in normal liver. Here, we report Western and Northern analyses detecting large quantities of AAAD polypeptide and mRNA in human carcinoid primary as well as metastatic tumors compared with normal surrounding tissues. To assess the feasibility of targeting these high AAAD levels for chemotherapy, AAAD inhibitors carbidopa ( $\alpha$ -methyl-dopahydrazine),  $\alpha$ -monofluoromethyl-dopa (MFMD), and 3-hydroxybenzylhydrazine (NSD-1015) were incubated (72 h) with NCI-H727 human lung carcinoid cells. Carbidopa and MFMD were lethal ( $IC_{50} = 29 \pm 2 \mu\text{M}$  and  $56 \pm 6 \mu\text{M}$ , respectively); NSD-1015 had no effect on proliferation. On exposure to other human tumor lines, carbidopa was lethal only to NCI-H146 and NCI-H209 small cell lung carcinoma (SCLC) lines ( $IC_{50} = 12 \pm 1 \mu\text{M}$  and  $22 \pm 5 \mu\text{M}$ , respectively). Carbidopa ( $100 \mu\text{M}$ ) decreased growth of (but did not kill) SK-N-SH neuroblastoma and A204 rhabdomyosarcoma cells and did not affect proliferation of DU 145 prostate, MCF7 breast, or NCI-H460 large cell lung carcinoma lines. The rank order of lines by AAAD activity was NCI-H146 > NCI-H209 > SK-N-SH > NCI-H727, whereas A204, DU 145, MCF7, and NCI-H460 had no measurable activity. For lung tumor lines (carcinoid, two SCLC, and one large cell lung carcinoma), AAAD activity was correlated with the potency of carbidopa-induced cytotoxicity. However, carcinoid cell death

was not solely attributable to complete inhibition of either AAAD activity or the serotonin synthetic pathway. In further evaluating potential applications of these findings with carbidopa, we determined that sublethal doses of carbidopa produced additive cytotoxic effects in carcinoid cells in combination with etoposide and cytotoxic synergy in SCLC cells when cocultured with topotecan.

## INTRODUCTION

The carcinoid tumor is the most frequently occurring neoplasm in the small bowel (1). Surgical resection is usually curative for primary small bowel lesions <2 cm, whereas larger primaries generally metastasize to the liver and are usually lethal. Furthermore, metastatic lesions in the liver are often associated with symptoms of the malignant carcinoid syndrome, including flushing, diarrhea, and eventually carcinoid heart disease. Among the molecules associated with these symptoms is 5-HT,<sup>3</sup> synthesized in the tumor from trp via the sequential action of TPH (EC 1.14.16.4) and AAAD (EC 4.1.1.28). The second most common site for carcinoid primaries is the lung (2), where large primary tumors can produce the carcinoid syndrome in the absence of metastases (3). In addition to the carcinoid neoplasm, other tumors are known to produce 5-HT and have high levels of AAAD activity, *e.g.*, SCLC (4, 5). There is currently no effective treatment for either carcinoid disease or SCLC at advanced stages.

In a previous study characterizing the enzyme kinetics of TPH and AAAD in human carcinoid hepatic metastases and adjacent normal liver, we determined that the  $K_m$  and  $V_{\max}$  of TPH and the  $K_m$  of AAAD were similar in carcinoid and normal tissue (6). In contrast, the  $V_{\max}$  for carcinoid AAAD was 50-fold higher than that of normal liver AAAD. In the present study, we investigated whether this difference was regulated at the transcriptional/translational level and whether it was characteristic of primary (ileal) carcinoid neoplasms in comparison with normal tissue as well as hepatic carcinoid metastases.

5-HT induces proliferation of a variety of cultured cells, including aortic endothelial and smooth muscle, pulmonary artery and cerebral vessel smooth muscle, and renal mesangial, lung fibroblast, pancreatic carcinoid, and small cell lung carcinoma cells (see reviews, Refs. 7 and 8). We examined AAAD inhibition as a possible antitumor therapeutic strategy and here report the effects of the AAAD inhibitors carbidopa ( $\alpha$ -methyl-

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<sup>3</sup> The abbreviations used are: 5-HT, 5-hydroxytryptamine or serotonin; trp, tryptophan; 5-OH-Trp, 5-hydroxytryptophan; TPH, trp hydroxylase; AAAD, aromatic-L-amino acid decarboxylase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MFMD,  $\alpha$ -monofluoromethyl-dopa; NSD-1015, 3-hydroxybenzylhydrazine; SCLC, small cell lung carcinoma; VP-16, etoposide; CI, combination index.

dopahydrazine), MFMD, and NSD-1015 on the proliferation of NCI-H727 pulmonary carcinoid cells. Carbidopa is typically coadministered with L-Dopa for the treatment of Parkinson's disease to inhibit peripheral AAAD. MFMD is a selective, enzyme-activated, irreversible inhibitor of AAAD (9); NSD-1015 is a commonly used AAAD inhibitor *in vitro*. Extending the above approach, we evaluated carbidopa exposure in a series of human tumor cell lines with a wide range of AAAD levels to determine the relationship between tumor cell AAAD activity and the effect of carbidopa on cell proliferation. Included in this series were SCLC cell lines (NCI-H146 and NCI-H209) with very high levels of AAAD activity (10, 11) and mRNA (11, 12). Preliminary accounts of this work have been presented in abstract form (13, 14).

## MATERIALS AND METHODS

**Materials.** Molecular biology grade reagents were purchased from Sigma (St. Louis, MO) except for: 50× Denhardt's solution (United States Biochemical, Cleveland, OH); agarose (FMC Bioproducts, Rockland, ME); 3 M sodium acetate (pH 5.2) and 10× MOPS (Amresco, Solon, OH); and AG 501-X8(D) (Bio-Rad, Hercules, CA). Poly(A) was purchased from Boehringer Mannheim (Indianapolis, IN); high-performance liquid chromatography grade methanol was from EM (Gibbstown, NJ) and *n*-propyl alcohol from Burdick & Jackson (Muskegon, MI). DTT, 5-HT, 5-OH-trp, carbidopa, NSD-1015, and VP-16 were purchased from Sigma Chemical Co. (St. Louis, MO). *N*-Methyl-*N*-propargylbenzylamine hydrochloride and triethylamine were obtained from Aldrich Chemical Co. (Milwaukee, WI), and pyridoxal-5-phosphate was from the Calbiochem Corp (San Diego, CA). Topotecan was provided by the National Cancer Institute; MFMD was a kind gift from Merrell Dow Research Institute (Cincinnati, OH). All of the other chemicals used were reagent grade.

**Human Tissues.** Specimens were flash-frozen after excision and stored at  $-70^{\circ}\text{C}$ .

**Western Analysis.** Preparation of tissue homogenates (100,000 × *g* supernatants), protein assays, and Western analyses were performed as described previously (6). Rabbit anti-AAAD antibody was supplied by Eugene Tech International, Inc. (Ridgefield Park, NJ).

**Northern Analysis.** A 40mer probe (5'-TTT GCC ATC TGT TCT CGC ACG GTG GAA TCT GCC CAT GTG C-3') complementary to coding sequence of human AAAD cDNA (Ref.15; GenBank accession no. M88700) was synthesized by Mayo Molecular Biology Core Facility.

Total RNA was isolated from frozen tissue with RNA Stat-60 reagent (Tel-Test "B"; Friendswood, TX). Poly(A)<sup>+</sup> RNA was then isolated with the Fast Track kit (Invitrogen, San Diego, CA) and quantitated by measuring  $A_{260}$ . RNA was electrophoretically fractionated in 1.2% agarose formaldehyde gels followed by downward capillary transfer (Turboblotter; Schleicher & Schuell, Keene, NH) in 20× SSC to Nytran nylon membranes (Schleicher & Schuell). After UV cross-linking, RNA was stained with methylene blue (16). The blots were prehybridized (24 h at 42°C) in: 5× SSC, 10× Denhardt's solution, 250 μg/ml denatured and sheared herring sperm DNA, 10 μg/ml poly(A), 0.1% SDS, and 50% deionized formamide.

The blots were hybridized with 25 × 10<sup>6</sup> cpm/ml of 40mer radiolabeled with [ $\alpha$ -<sup>32</sup>P]dATP (DuPont NEN, Boston, MA) with the DNA Tailing Kit (Boehringer Mannheim) and purified on a NENSORB 20 cartridge (DuPont NEN). Posthybridization washes were: 5× SSC (twice for 2 min at 24°C); 2× SSC, 0.1% SDS (four times for 5 min at 24°C); and 1.0× SSC, 0.1% SDS (three times for 15 min at 45°C). Autoradiography was for 4–10 days at  $-70^{\circ}\text{C}$ .

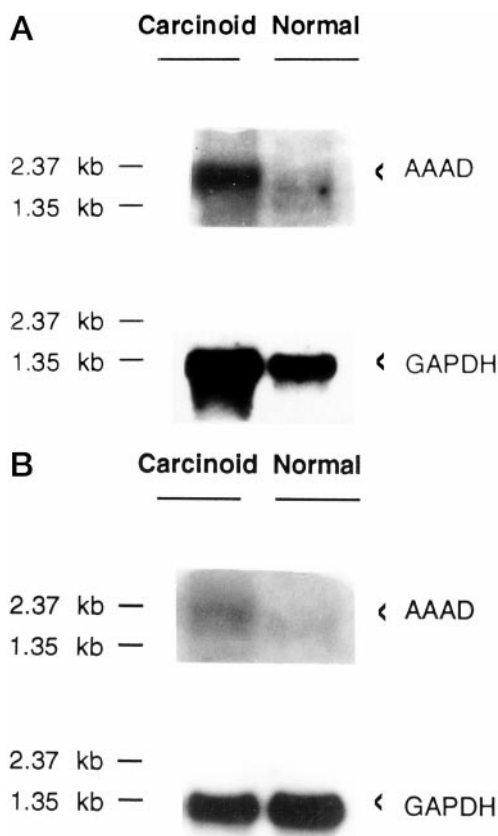
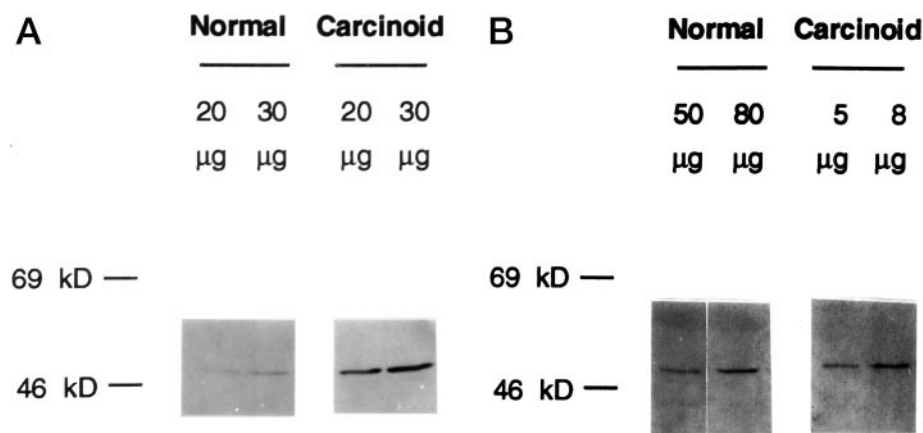
After removal of the probe with 2× saline-sodium phosphate-EDTA buffer, 55% formamide, 1% SDS (1 h at 65°C), the blot was prehybridized and reprobbed as above with 5 × 10<sup>6</sup> cpm/ml of a 1.0-kb human GAPDH cDNA fragment (Clontech, Palo Alto, CA) radiolabeled with [ $\alpha$ -<sup>32</sup>P]dCTP (Amersham, Arlington Heights, IL) with the Rediprime DNA Labeling System (Amersham) and purified on a NENSORB 20 cartridge. Posthybridization washes were: 5× SSC (twice for 2 min at 24°C); 2× SSC, 0.1% SDS (four times for 5 min at 24°C); and 0.1× SSC, 0.1% SDS (three times for 15 min at 50°C). Autoradiography was for 1–6 days at  $-70^{\circ}\text{C}$ .

**Cell Culture.** All of the cell lines were purchased from American Type Culture Collection (Manassas, VA), and cultured at 37°C in a humidified environment of 95%:5% air:CO<sub>2</sub> in the following media (Life Technologies, Inc., Grand Island, NY) supplemented with 10% qualified fetal bovine serum (Life Technologies, Inc.): NCI-H146, -H209, -H727, and -H460 lines in RPMI 1640; A204 and MCF7 in DMEM; and Du 145 and SK-N-SH in MEM with Earle salts, 1 mM sodium pyruvate, and 2 mM L-glutamine.

**Trypan Blue Exclusion Assays.** Cells (50,000) were seeded in 60-mm plates (Falcon, Lincoln Park, NJ) in 3 ml of growth medium supplemented with 50 units/ml of penicillin and streptomycin (Life Technologies, Inc.). After incubation overnight, the appropriate AAAD inhibitor concentration or diluent was added to the plate of a suspension cell line or within 3 ml of fresh media to a monolayer cell line (20 mM stock inhibitor solutions were prepared in DMSO and stored at 4°C before use). For continuous exposure, drug was replaced daily. Treated cells were collected by centrifugation for 5 min at 4°C and 200 × *g*: suspension cell lines, directly; and attached cell lines, on combination of media with monolayer cells lifted with 0.05% trypsin, 0.53 mM EDTA (Life Technologies, Inc.). After resuspension in culture medium, viable cells were counted on a hemocytometer after a 5-min incubation in 0.2% trypan blue (Life Technologies, Inc.). IC<sub>50</sub> values were calculated from dose-response plots by the method of nonlinear least-squares regression to a logistic function. These values represented the concentration of inhibitor required to bring the number of viable, treated cells to 50% of the control cell number upon 72 h of incubation. Data from experiments assessing combined drug effects were analyzed by the median effect method of Chou and Talalay (17), both as though drug actions were mutually non-exclusive (*i.e.*, the mechanisms were independent of one another) as well as though they were mutually exclusive.

**Cell Homogenates.** Homogenates were prepared at 4°C. Suspension cells were washed twice with PBS (Life Technologies, Inc.) prior to collection by centrifugation; monolayer cells were detached from flasks after washing by scraping (Nunc 179707; Rochester, NY) into PBS prior to collection. A volume of 0.3 M Tris-acetate, 5 mM DTT (pH 7.6) equal to the weight of

**Fig. 1** Western analysis of AAAD. *Upper numbers*, the quantity of homogenate protein per sample ( $\mu\text{g}$ ). Data are representative of experiments analyzing three matched sets of (A) hepatic carcinoid metastasis and adjacent normal liver, and (B) primary ileal carcinoid tumor and adjacent normal ileum.



**Fig. 2** Northern analysis of AAAD. Data are representative of experiments analyzing three matched sets of (A) hepatic carcinoid metastasis and adjacent normal liver (7  $\mu\text{g}$  mRNA) from the patient set in Fig. 1A, and (B) primary ileal carcinoid tumor and adjacent normal ileum (9  $\mu\text{g}$  mRNA) from one patient set.

the cellular pellet was added prior to probe-sonication (Braun-sonic 1510; B. Braun, Melsungen, Germany) at 100 W for two 15-s bursts on ice. After ultracentrifugation at  $100,000 \times g$  for 1 h, the supernatant was divided into aliquots and stored at  $-70^\circ\text{C}$  until use.

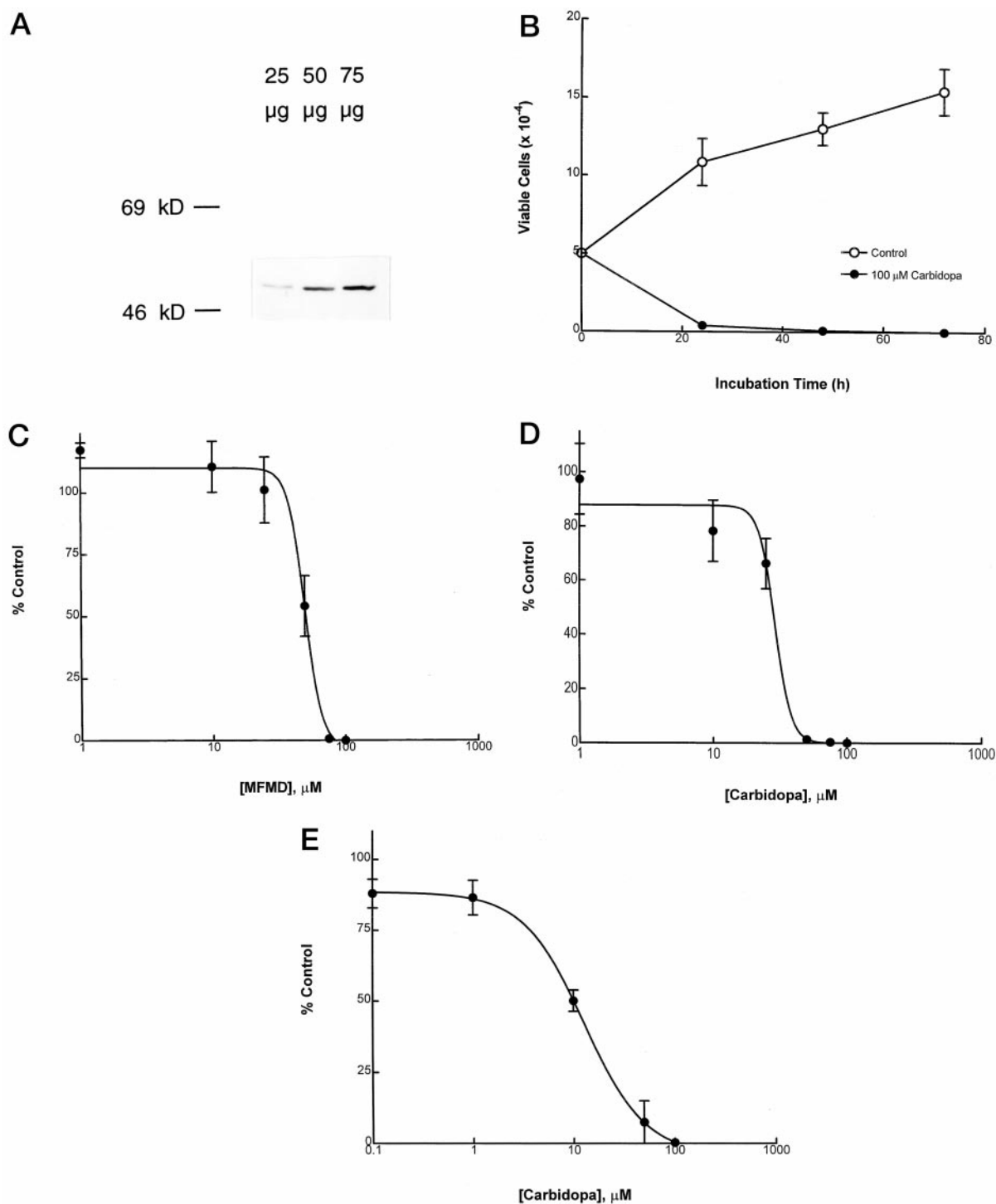
**AAAD Assay.** The assay conditions described previously (6) were used for homogenates.

To measure AAAD activity in intact cells after exposure to an AAAD inhibitor, cells (50,000) were seeded in 60-mm plates in 3 ml of growth medium, treated with the appropriate AAAD inhibitor concentration as described above, and incubated at  $37^\circ\text{C}$  for the appropriate length of time. Cells from six replicate plates were collected by centrifugation, monolayer cells first being lifted with 0.05% trypsin, 0.53 mM EDTA. One hundred fifty  $\mu\text{l}$  of 0.3 M Tris-acetate (pH 7.6) was added prior to probe-sonication; the broken cell preparation was then assayed for activity as described above.  $\text{IC}_{50}$  values were calculated from dose-response plots as the concentration of inhibitor required to bring the activity in homogenate from treated cells to 50% of that in control cell homogenate upon 10 min of incubation prior to cell harvesting.

**Electron Microscopy.** NCI-H727 carcinoid cells were plated as for trypan blue assays and incubated in experimental medium, alone or with 25  $\mu\text{M}$  carbidopa for 24 h. Adherent and nonadherent cells were then collected separately, washed in PBS, and resuspended in Truumps fixative [1% glutaraldehyde and 4% formaldehyde in 0.1 M phosphate buffer (pH 7.2)]. Samples were postfixed in phosphate-buffered 1%  $\text{OsO}_4$  and stained with 2% uranyl acetate. Samples were dehydrated and embedded in Spurr's resin. Sections (90 nm) were cut on a Reichert Ultracut E ultramicrotome, placed on 200 mesh copper grids, and stained with lead citrate. Micrographs were taken on a JEOL 1200 EXII electron microscope.

## RESULTS

**Analysis of AAAD in Hepatic Carcinoid Metastases and Adjacent Normal Liver Samples.** Our previous studies (6) indicated, somewhat surprisingly, that AAAD activity was significantly increased in carcinoid tumor compared with surrounding normal tissue. To evaluate the basis for this increase, we examined levels of AAAD polypeptide by immunoblotting and AAAD message by Northern analysis in a series of matched tissue sets. Western analysis with anti-AAAD antibody revealed a single band with a mobility pattern consistent with the reported  $M_r$  50,000 of human pheochromocytoma AAAD on SDS-PAGE (18). Fig. 1A illustrates representative data from



**Fig. 3** A, Western analysis of AAAD in NCI-H727 carcinoid cells. *Upper numbers*, the quantity of homogenate protein per sample ( $\mu$ g). Data represent one of triplicate experiments. B, time course of carbidopa effect on NCI-H727 carcinoid cell number. Cells (50,000/plate) were incubated at 37° with carbidopa (continuous exposure). At the indicated times, the number of viable cells in each plate was determined with the trypan blue exclusion assay described in “Materials and Methods.” These data represent the average  $\pm$  SE of triplicate experiments. C, effect of increasing concentrations of MFMD on NCI-H727 carcinoid cell number. Cells (50,000/plate) were incubated at 37° with MFMD (continuous exposure, 72 h). The number of viable cells in each plate was determined with the trypan blue exclusion assay. The average number of viable cells in duplicate experimental plates was normalized to the average number of viable cells in triplicate control plates. These data represent the average  $\pm$  SE of triplicate experiments. D, effect of increasing concentrations of carbidopa on NCI-H727 carcinoid cell number. Experiments were performed and calculated as in Fig. 3C. These data represent the average  $\pm$  SE of triplicate experiments. E, effect of increasing concentrations of carbidopa on NCI-H727 AAAD activity. Cells (50,000/plate) were incubated at 37° with carbidopa for 10 min before determination of AAAD activity in broken-cell preparations. These data represent the average  $\pm$  SE of triplicate experiments.

one of three matched sets of hepatic carcinoid metastases and surrounding normal liver tissues. AAAD bands on Western blots were markedly enhanced in the tumors compared with the adjacent normal liver samples.

When the same samples of normal liver were subjected to Northern analysis with a [<sup>32</sup>P]-40mer probe complementary to AAAD coding sequence, no AAAD mRNA was detected. However, a major band of ~2.1 kb, consistent with the reported size of AAAD message in human pheochromocytoma (19), was easily detected in mRNA isolated from the three respective hepatic carcinoid metastases. Representative data from one matched set of tissues, the same pair examined by immunoblotting in Fig. 1A, are illustrated in Fig. 2A. Some nonspecific binding of the 40mer probe to residual 18S and 28S rRNA in the mRNA preparations was also seen. Reprobing of the blot with a [<sup>32</sup>P]-labeled GAPDH cDNA fragment confirmed the absence of mRNA degradation and was used for normalizing (via AAAD:GAPDH densitometric ratios) the mRNA loading in each lane.

**Analysis of AAAD in Primary Ileal Carcinoid Tumors and Adjacent Normal Ileum Samples.** To determine whether AAAD levels were increased in primary carcinoid lesions when compared with surrounding normal tissue (as was observed with metastatic hepatic lesions), primary ileal carcinoid tumors and surrounding normal ileum samples were examined by Western blotting (Fig. 1B). The AAAD polypeptide level in the tumors was markedly higher than in their adjacent normal tissues ( $n = 3$ ). Because the tissue quantity obtained in pairs of primary ileal carcinoid tumors and surrounding normal ileum was limited, Northern analysis was performed in a different triplicate of matched tissue sets. No AAAD mRNA was detectable in two normal ileum samples, and a very weak ~2.1-kb band was detected in the third. A major band consistent with human AAAD mRNA was readily detected in all of the three respective primary ileal carcinoid tumors (Fig. 2B).

**Effect of Exposure to AAAD Inhibitors on Proliferation and Intracellular AAAD Activity of Lung Carcinoid Cells.** The NCI-H727 human lung carcinoid line has abundant AAAD activity (8) and polypeptide content (Fig. 3A). To assess the effect of AAAD inhibition on carcinoid tumor growth, three AAAD inhibitors, carbidopa, MFMD, and NSD-1015, were incubated with NCI-H727 cells for 72 h, and the loss of viability was determined by trypan blue uptake assays. Carbidopa and MFMD were cytotoxic to the carcinoid cells, whereas NSD-1015 had no effect on growth. A time course of cytotoxicity induced by 100  $\mu\text{M}$  carbidopa (Fig. 3B) indicated that carcinoid cell death was detectable at 24 h, whereas total cell kill occurred at 48–72 h. Dose-response curves of MFMD- and carbidopa-induced cell death in the carcinoid cell line are shown in Fig. 3, C and D. Table 1 summarizes cytotoxicity data at 72 h on carcinoid cells exposed to the three AAAD inhibitors. For comparison of this effect with that of a known chemotherapeutic agent, NCI-H727 carcinoid cells were exposed to doxorubicin, lethal to sensitive human tumor cell lines at  $\text{nm IC}_{50}$  values (20). In contrast, doxorubicin ( $\leq 100 \mu\text{M}$ ;  $n = 2$ ) was not lethal to NCI-H727 cells.

To examine the relationship between the effect on carcinoid cell growth of carbidopa, MFMD, and NSD-1015 and their inhibition of intracellular AAAD, enzyme activity assays were

Table 1 Effect of AAAD inhibitors on intact NCI-H727 carcinoid cells<sup>a</sup>

Inhibitor	Cytotoxicity (IC <sub>50</sub> , $\mu\text{M}$ )	AAAD inhibition (IC <sub>50</sub> , $\mu\text{M}$ )
Carbidopa	29 $\pm$ 2	16 $\pm$ 5
MFMD	56 $\pm$ 6	2 $\pm$ 0.3
NSD-1015	None detected	Incomplete

<sup>a</sup> Cells (50,000/plate) were incubated continuously at 37°C with increasing concentrations of inhibitor ( $\leq 100 \mu\text{M}$ ) for 72 h prior to cytotoxicity measurement by trypan blue exclusion or for 10 min before determination of AAAD activity in broken-cell preparations (average  $\pm$  SE;  $n = 3$ ).

performed on broken-cell preparations of carcinoid cells after incubation of whole cells with increasing concentrations of inhibitor. Carbidopa and MFMD (100  $\mu\text{M}$ ) totally inhibited intracellular AAAD activity within 10 min of exposure to intact NCI-H727 cells at 37° (see Table 1 and carbidopa-induced AAAD inhibition in Fig. 3E). At the identical concentrations and exposure times, NSD-1015 did not completely inhibit intracellular carcinoid AAAD activity, nor was it lethal to NCI-H727 cells. However, when solubilized directly in media, NSD-1015 could be dissolved at 1000  $\mu\text{M}$ . At this concentration, NSD-1015 totally inhibited AAAD activity in carcinoid cells ( $n = 2$ ) but still had no growth inhibitory effect ( $n = 2$ ). Because AAAD inhibition would block the biosynthesis of 5-HT, we further examined a possible role for 5-HT and the precursor 5-OH-trp in the cytotoxicity of AAAD inhibitors. No effect on carcinoid cell proliferation was produced by the addition of the AAAD substrate 5-OH-trp, which presumably would accumulate in cells as a consequence of AAAD inhibition ( $\leq 100 \mu\text{M}$ ;  $n = 2$ ). Furthermore, the specific TPH inhibitor *p*-chlorophenylalanine did not alter NCI-H727 cell growth ( $\leq 1000 \mu\text{M}$ ;  $n = 2$ ).

**Effect of Carbidopa on Proliferation of Human Tumor Cell Lines Compared with the Maximal AAAD Activity of the Cell Lines.** The most potent cytotoxic AAAD inhibitor, carbidopa, was incubated with seven additional human tumor cell lines exhibiting a wide range of AAAD activity levels. Micromolar concentrations of carbidopa were lethal to three of eight lines, all of lung origin: SCLC NCI-H146, SCLC NCI-H209, and carcinoid NCI-H727 cells (Table 2). A moderate decrease in cellular growth (but not complete cell death) was observed in the SK-N-SH neuroblastoma and A204 rhabdomyosarcoma tumor cell lines, whereas no effect was observed in the remaining three cell lines. Table 2 also summarizes the maximal AAAD activity determined in homogenates from all of the eight human tumor cell lines. The relationship between the potency of carbidopa-induced cytotoxicity and the maximal AAAD activity for the four lung tumor lines is illustrated in Fig. 4.

**Electron Microscopy of Carbidopa-treated Carcinoid Cells.** Compared with control carcinoid cells (Fig. 5A), NCI-H727 cells treated with 25  $\mu\text{M}$  carbidopa for 24 h consisted of normal-looking cells as well as cells having a “foamy” appearance resulting from the presence of rounded vacuoles and/or loss of cytoplasmic integrity (Fig. 5B). These carbidopa-treated cells maintained relatively normal nuclear morphology and intact plasma membranes.

Table 2 Human tumor cell lines: carbidopa sensitivity and maximal AAAD activity<sup>a</sup>

Human tumor cell line		Cell survival on carbidopa exposure	Maximal AAAD activity (nmol/min/mg protein)
NCI-H146	SCLC	0% (IC <sub>50</sub> = 12 ± 1 μM) <sup>b</sup>	2.329 ± 0.086 <sup>c</sup>
NCI-H209	SCLC	0% (IC <sub>50</sub> = 22 ± 5 μM) <sup>b</sup>	0.83 ± 0.048 <sup>c</sup>
NCI-H727	Carcinoid	0% (IC <sub>50</sub> = 29 ± 2 μM) <sup>b</sup>	0.342 ± 0.085 <sup>c</sup>
SK-N-SH	Neuroblastoma	27% <sup>d</sup>	0.658 ± 0.083 <sup>c</sup>
A204	Rhabdomyosarcoma	42% <sup>d</sup>	None detected <sup>c</sup>
MCF7	Breast carcinoma	100% <sup>d</sup>	None detected <sup>c</sup>
NCI-H460	Large cell lung carcinoma	100% <sup>d</sup>	None detected <sup>e</sup>
Du 145	Prostate carcinoma	100% <sup>d</sup>	None detected <sup>e</sup>

<sup>a</sup> Carbidopa (≤100 μM; 72 h exposure) cytotoxicity was determined by trypan blue exclusion and AAAD activity levels by the AAAD assay of cellular homogenates.

<sup>b</sup> Average ± SE; n = 3.

<sup>c</sup> Average ± SE; n ≥ 3.

<sup>d</sup> Carbidopa concentration, 100 μM; n = 2; percentage survival = no. of viable treated cells/no. of viable control cells.

<sup>e</sup> n ≥ 2.

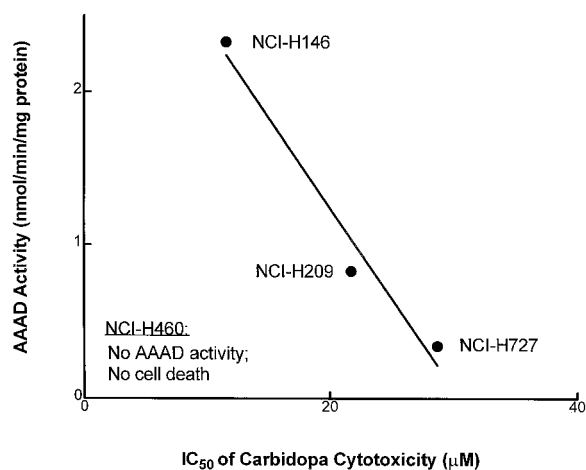


Fig. 4 Correlation of the IC<sub>50</sub> of carbidopa cytotoxicity with the maximal AAAD activity of human lung tumor cell lines.

**Effect of Carbidopa Coincubation with Chemotherapeutic Drugs on Proliferation of Carcinoid and SCLC Cells.** To assess the potential of carbidopa for use in chemotherapy, it was coincubated at sublethal doses with known chemotherapeutic agents to assess the effect on the proliferation of the NCI-H727 carcinoid and NCI-H146 SCLC cell lines. VP-16 alone (72 h) was not lethal to carcinoid cells, although it did decrease cellular growth (Fig. 6A). However, a 1:30 combination of VP-16-with-carbidopa induced carcinoid cell death. Analysis of these combination experiments with the median effect method of Chou and Talalay (17) indicated that the cytotoxic effects on carcinoid cells of VP-16 and carbidopa coincubation were additive (data not shown). Topotecan alone decreased the growth of SCLC cells (Fig. 6B). When combined 1:3000 with carbidopa, the cytotoxic effect was greater than that produced by either drug alone. Analysis of these results with the median effect method (Fig. 6C) indicated that topotecan and carbidopa acted synergistically (CI < 1) in SCLC cells when the fraction of cell kill was >65%.

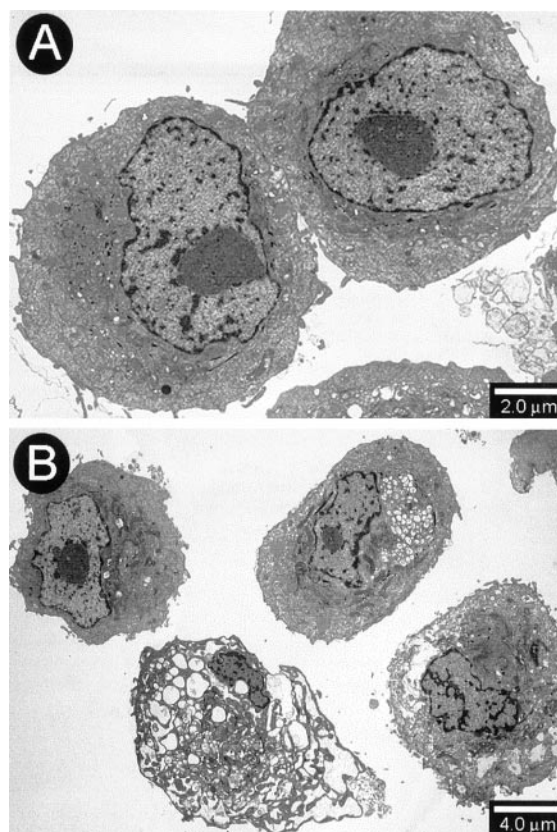
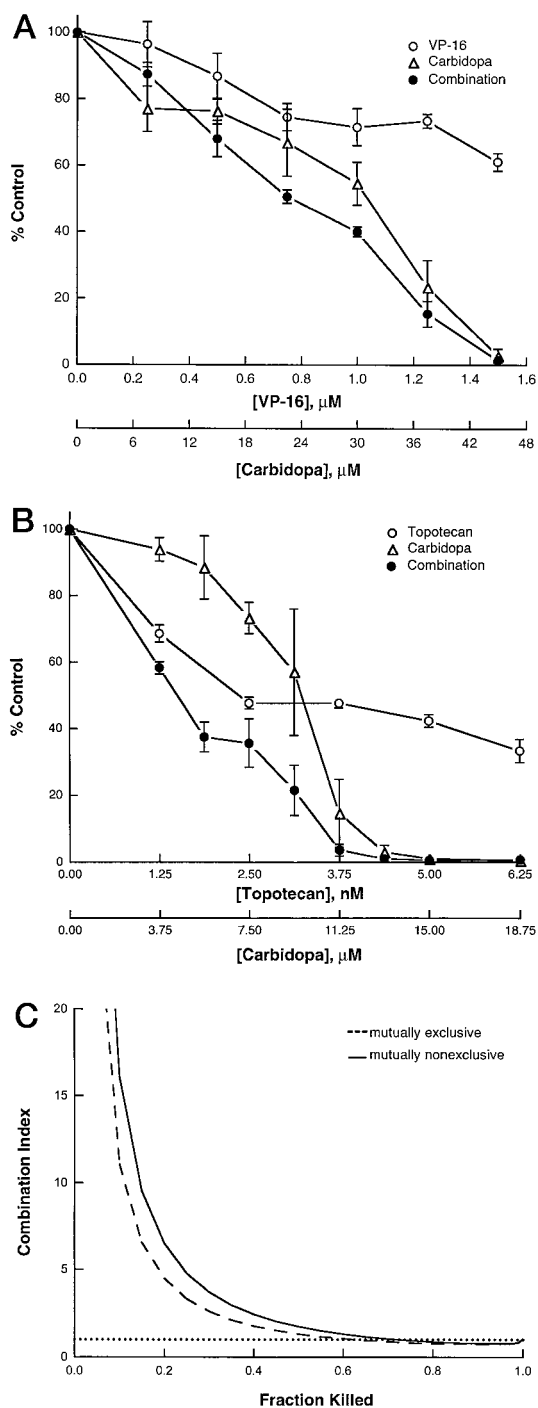


Fig. 5 Transmission electron micrographs of NCI-H727 carcinoid cells. A, control cells in culture medium alone (24 h) were intact and showed no obvious signs of cell death. B, a population of nonadherent cells exposed to 25 μM carbidopa (24 h) contained rounded vacuoles and/or a loss of cytoplasmic integrity.

## DISCUSSION

This study focuses on AAAD, the second enzyme of the 5-HT biosynthetic pathway, which, in carcinoid hepatic metastases, displays high levels of activity compared with that in surrounding normal tissue (6). Here we describe the large quantities of AAAD



**Fig. 6** A, effect of increasing concentrations of VP-16, carbidopa, and a combination of both on NCI-H727 carcinoid cell number. Experiments were performed and calculated as in Fig. 3C, with coincubations performed at a constant 1:30 ratio of VP-16:carbidopa. These data represent the average  $\pm$  SE of quadruplicate experiments. B, effect of increasing concentrations of carbidopa, topotecan, and a combination of both on NCI-H146 SCLC cell number. Experiments were performed and calculated as in Fig. 3C, with coincubations performed at a constant 1:3000 ratio of topotecan:carbidopa. These data represent the average  $\pm$  SE of triplicate experiments. C, CI plot for the coincubation of topotecan and carbidopa with NCI-H146 SCLC cells. Analysis was based on data in B; correlation coefficients were  $>0.95$  for all of the three median effect lines in these calculations. Dotted line: CI = 1.

polypeptide found in both hepatic carcinoid metastases and primary ileal lesions but not in surrounding normal tissues. Furthermore, high levels of AAAD mRNA were detected in metastatic and primary tumors, whereas low-to-undetectable levels were found in respective normal tissues. The inability to measure AAAD mRNA in the majority of normal tissue samples examined was not unexpected, given the very low abundance of AAAD mRNA (from 0.0005% of total RNA in brain to 0.0035% of total RNA in kidney) found in normal rat tissues (21). Collectively, these results indicate that the increased AAAD activity observed in carcinoid tumors reflected increased steady-state mRNA levels.

Historically, AAAD was believed to be an unregulated enzyme present in excess of TPH in serotonergic tissues and in substantial quantities in nonserotonergic neurons and in peripheral organs, in which the function of the enzyme is not fully understood. Our studies, demonstrating high levels of AAAD protein and mRNA in human carcinoid primary and metastatic tumors relative to surrounding normal tissue, are consistent with recent evidence suggesting that AAAD may undergo transcriptional, translational, and posttranslational regulation (see reviews, Refs. 22 and 23).

In assessing tumor AAAD as a selective target for chemotherapeutic agents, we found that the AAAD inhibitors carbidoapa and MFMD were lethal to NCI-H727 human lung carcinoid cells. This strategy was extended to a number of human tumor cell lines with varying amounts of cellular AAAD. The comparison of the  $\text{IC}_{50}$  value for carbidoapa cytotoxicity with AAAD activity in each cell line indicated that, in general, cell lines with higher AAAD levels were more susceptible to carbidoapa-induced cytotoxicity. The two exceptions were the neuroblastoma SK-N-SH line, which did not exhibit complete carbidoapa-induced cell kill although having relatively high levels of AAAD activity, and the rhabdomyosarcoma A204 line, which demonstrated a moderate response to carbidoapa although having no detectable AAAD. Carbidoapa was lethal to only three lung lines (the NCI-H727 carcinoid line and the NCI-H146 and NCI-H209 SCLC lines), all three with abundant AAAD activity. NCI-H146 and NCI-H209 SCLC cells, in fact, have among the highest AAAD mRNA levels of the 38 (12) and 27 (10) neuroendocrine lines compared in the literature. We found a direct correlation between the potency of carbidoapa-induced cytotoxicity and level of AAAD activity in the lung tumor lines, including NCI-H460 large cell lung carcinoma, which lacked AAAD activity and was unresponsive to carbidoapa. Carbidoapa was not lethal to the non-lung tumor lines tested.

There are several potential mechanisms for carbidoapa- (and MFMD-) induced cytotoxicity to carcinoid and SCLC cells. Through AAAD inhibition, carbidoapa might inhibit formation of a factor required for the growth/survival of these cells, *e.g.*, 5-HT. However, no cytotoxicity to carcinoid cells was observed after complete inhibition of AAAD by NSD-1015, and no effect on growth was observed on incubation of these cells with TPH inhibitor, *p*-chlorophenylalanine. Furthermore, apoptotic cell death is often correlated with an interruption of cell-signaling pathways critical to cell survival. Investigation of carcinoid cells exposed to carbidoapa (25  $\mu\text{M}$  for 24 h) did not reveal, by Hoechst staining or transmission electron microscopy, the morphological changes typically associated with either apoptosis or necrosis. Thus, the 5-HT biosynthetic pathway appears not to be the mechanism of carbidoapa-induced cell death.

Another possible mechanism might involve AAAD inhibi-

tion directly without relation to any associated effect on 5-HT synthesis. However, some of our results suggest that the relationship between AAAD expression and inhibitor cytotoxicity is not straightforward. Two of the eight cell lines did not exhibit a direct relationship between AAAD content and susceptibility to carbidopa-induced cell death (neuroblastoma SK-N-SH and rhabdomyosarcoma A204). In addition, of the two toxic AAAD inhibitors, carbidopa was more potent in inducing carcinoid cell death, whereas MFMD was the better AAAD inhibitor. Finally, carbidopa did not completely inhibit AAAD activity in intact NCI-H146 SCLC cells at concentrations that were lethal to the cells (data not shown), whereas concentrations of NSD-1015 high enough to shut down AAAD activity in intact NCI-H727 cells did not cause death in these cells, which indicated that complete inhibition of AAAD was not required for carbidopa-induced cell death in the carcinoid and at least one SCLC line.

Mechanisms other than interruption of 5-HT synthesis or direct inhibition of AAAD may well be responsible for the selective cytotoxicity of carbidopa. Carbidopa may be metabolized to a toxic product by certain cell lines with high levels of AAAD. Interestingly, a previous report indicated that Chinese hamster ovary fibroblasts transfected with bovine AAAD cDNA had increased intracellular catechol levels and increased sensitivity to the lethal combination of L-Dopa and manganese relative to wild type fibroblasts. This was attributed to AAAD decarboxylation of intracellular L-Dopa accumulated from growth medium to form dopamine, the proposed protoxin for manganese-induced intracellular oxidation (24). Our data suggest that high levels of AAAD might be a necessary although not sufficient requirement for lung tumor lines to be susceptible to carbidopa-induced cytotoxicity, with abundant AAAD perhaps serving only as a marker for cells sensitive to this effect.

The selective carbidopa cytotoxicity toward human pulmonary carcinoid and SCLC cell lines suggests a possible new therapeutic approach for these two neoplasms, both presently untreatable in advanced stages. To further capitalize on these findings, we conducted studies of carbidopa in combination with several classes of antitumor agents. Preliminary data were most favorable for topoisomerase inhibitors (data not shown). The combination of VP-16 and carbidopa was cytotoxic in an additive fashion to carcinoid cells, a tumor generally unresponsive to traditional chemotherapeutic drugs (1, 3). In addition, sublethal concentrations of carbidopa (closer to those typically administered in Parkinson's disease), displayed cytotoxic synergy in combination with topotecan in SCLC cells. The mechanism of carbidopa-induced cell killing, alone and in combination with known chemotherapeutic drugs, and the role played by AAAD expression and carbidopa metabolism in this cytotoxicity are currently under further investigation.

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