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BIOCHEMICAL PHARMACOLOGY OF *N*-ACETYL-*N*-(METHYLCARBAMOYLOXY)-*N'*-METHYLUREA (CARACEMIDE; NSC-253272)*

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Abstract—Preclinical pharmacologic studies of caracemide [*N*-acetyl-*N*-(methylcarbamoyloxy)-*N'*-methylurea; CAR] have demonstrated a marked instability of this compound in the presence of either phosphate buffer (pH 7.4) or human plasma. Using [1-^{14}C-*acetyl*]CAR and [^{3}H-*methyl-carbamoyloxy*]CAR, three CAR degradation products were identified: product A, *N*-(methylcarbamoyloxy)acetamide; product B: *N*-(methylcarbamoyloxy)-*N'*-methylurea; and product C: *N*-hydroxy-*N'*-methylurea. CAR degradation in human plasma was demonstrated by high-performance liquid chromatography (HPLC) to occur in a time- and temperature-dependent manner. A 30-min incubation (37°) of CAR (10^{-4} M) with human plasma resulted in degradation of more than 55% of parent compound; at 1 hr, more than 75% of original CAR was degraded. Incubation of [1-^{14}C-*acetyl*]CAR with rat brain homogenate resulted in the formation of $^{14}CO_2$. This reaction was partially inhibited by coincubation with physostigmine (10^{-3} M). CAR inhibited acetylcholinesterase activity in neuroblastoma cells with an IC_{50} of 14 μM. In mechanism of action studies, CAR was found to inhibit ribonucleotide reductase activity but only at nine times the IC_{50} of hydroxyurea. In contrast to hydroxyurea, CAR was found to be non-cell-cycle phase-specific and non-cross-resistant with two CHO cell lines resistant to hydroxyurea. These data demonstrate the instability of CAR; moreover, they suggest that its mechanism of cytotoxicity is distinctly different from that of hydroxyurea and that the neurotoxicity associated with CAR administration may be caused in part by inhibition of acetylcholinesterase activity.

Caracemide [*N'*-acetyl-*N*-(methylcarbamoyloxy)-*N'*-methylurea; CAR; NSC-253272] (Fig. 1) is an antitumor agent synthesized by the Dow Chemical Co. from acetohydroxamic acid and methyl isocyanate. Its oncolytic activity against subrenal capsule implants of human MX-1 mammary and colon tumor xenografts, as well as against intraperitoneally implanted murine P388 leukemia cells, has been reported [1]. Initial studies to determine the mechanism of action of this novel agent showed that CAR inhibits macromolecular synthesis in P388 lymphocytic leukemia cells, the synthesis of DNA being

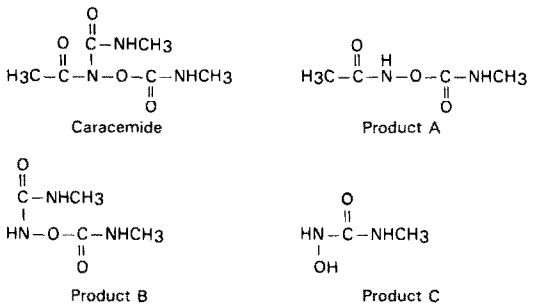

Fig. 1. Chemical structures of caracemide and its degradation products. Experimental conditions for the formation of degradation products are described in Materials and Methods. Identities of compound structures were determined by NMR, mass spectrometry, and chromatography.

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inhibited preferentially to that of RNA or protein [1]. Although DNA single-strand breaks are produced by CAR at concentrations of 100 μM or greater, lower concentrations of the drug are not associated with DNA lesions. The exact mechanism of action of this antitumor agent therefore remains unclear.

Clinical Phase 1 trials of CAR conducted at The University of Texas, M. D. Anderson Hospital and Tumor Institute, demonstrated that neurotoxicity is associated with intravenous administration of CAR doses exceeding 650 mg/m^2 [2]. The clinical symptoms of treated patients suggested a drug-mediated interference with the cholinergic nervous system [2]. The following studies were undertaken, therefore, to help elucidate certain aspects of the biochemical pharmacology and toxicology of CAR.

MATERIALS AND METHODS

N - [1 - ^{14}C - *acetyl*] - *N* - methylcarbamoyloxy - *N'* -methylurea (i.e. [1-^{14}C-*acetyl*]CAR; 12.1 mCi/mmole) and nonradioactive CAR were obtained from the Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD. Its radiochemical purity was greater than 97% as assessed by silica gel F thin-layer chromatography using toluene–acetone (1:1) as mobile phase. [^{14}C]Acetylcholine iodide (5 mCi/mmole), [^{3}H-*methyl*]iodide (100 mCi/mmole), [^{14}C]L-ornithine (250 mCi/mmole) and [^{3}H]cytidine 5'-diphosphate (20 mCi/mmole) were obtained from the New England Nuclear Corp., Boston, MA. All other chemicals and reagents were purchased from the usual commercial sources except for glass-distilled high-performance liquid chromatography (HPLC)-grade solvents supplied by Burdick and Jackson Laboratories, Muskegon, MI.

Proton magnetic resonance spectra (^{1}H NMR) of CAR and its degradation products were recorded at ambient temperature on a Varian Associates T-60A spectrometer in $CDCl_3$ or $(CD_3)_2SO$, using tetramethylsilane as an internal standard, or in D_2O using sodium 3-(trimethylsilyl)-1-propanesulfonate as an internal standard. Mass spectra were obtained on a Finnigan model 3300 quadrupole spectrometer in the electron impact mode with a direct inlet probe. The homogeneity of CAR and its degradation products was determined by ascending TLC on silica-coated glass plates (silica gel 60 F-254, Merck) using mixtures of chloroform and methanol as eluting solvent. The compounds were visualized by placing the air-dried chromatograms in a tank of iodine vapor. Unless otherwise specified, preparative separations were performed on columns of silica gel (70–230 mesh, Woelm) or on glass plates (20 × 20 cm) coated with a 2-mm layer of silica gel F254 (Merck).

Synthesis of N-*acetyl*-N-[^{3}*H*-methylcarbamoyloxy]-N'-*methylurea*. Since CAR labeled in the methylcarbamoyloxy group was unavailable commercially, it was synthesized in our laboratory as follows. A breakseal ampule of [^{3}H-*methyl*]iodide (25 mCi, 100 mCi/mmole) was partially immersed in a dry ice/acetone bath at −78° for 1 hr. The vial was then quickly transferred to a dry nitrogen glove box and anhydrous 1,2-dimethoxymethane (2.0 ml) was added to the reservoir at the top of the ampule. The seal was broken with a glass rod and the solvent passed quickly into the ampule. The solution was warmed to room temperature with occasional agitation to ensure thorough mixing. A 1.0-ml aliquot of this solution (which contained 17.75 mg of [^{3}H-*methyl*]iodide, 12.5 mCi) was transferred to a 5-ml round-bottomed flask fitted with a ground-glass stopper. Unlabeled methyl iodide (70.1 μl, 159.8 mg) was added, followed by silver isocyanate (187.5 mg, 1.25 mmoles), and the mixture was stirred in the dark for 24 hr. The reaction product was tranferred to a 5-ml screw-capped tube, which was centrifuged at 5000 *g* for 5 min to sediment silver iodide and unreacted silver isocyanate. The clear supernatant fraction was transferred to another 5.0-ml flask, and acetohydroxamic acid (94 mg, 1.25 mmoles) was added. The mixture was stirred for 24 hr and evaporated to dryness. The residue was taken up in methanol, then preadsorbed on silica gel (400 mg) and transferred to the top of a column (20 × 1 cm) of silica (Merck, 270–400 mesh) that had been made up in chloroform. The products were eluted with chloroform–methanol (6:1, v/v) using a mechanical pump operating at a back pressure of 40 lb/sq. m. Fractions (5 ml) were collected, and 50-μl aliquots of each were analyzed by TLC using iodine vapor to locate the products. Fractions containing radioactivity that comigrated with an authentic sample of *N*-(methylcarbamoyloxy)acetamide were combined and evaporated to yield a white solid weighing 10.5 mg. The radiochemical purity of a sample of this product was determined by TLC on silica using chloroform–methanol (6:1, v/v) as eluent. More than 97% of the radioactivity was observed to co-migrate with an authentic sample of *N*-(methylcarbamoyloxy)acetamide. The product was suspended in 5 ml of chloroform, and 30 mg of unlabeled *N*-(methylcarbamoyloxy)acetamide was added, followed by methyl isocyanate (19 μl, 0.34 mmole). The mixture was stirred at room temperature for 48 hr and then evaporated under nitrogen. The residue was taken up in methanol, preadsorbed on 300 mg of silica, and then transferred to a silica column (20 × 0.5 cm) made up in chloroform. The column was eluted with chloroform–methanol (6:1, v/v); 4-ml fractions were collected. [^{3}H]CAR was eluted in the first few fractions, which were combined and evaporated. The product was chromatographically identical to an authentic sample of CAR by TLC and HPLC analyses. The yield was 37 mg (64%). The radiochemical purity of the product was 98.6%, and the specific activity was 2.53 mCi/mmole.

CAR degradation in aqueous buffer. CAR is unstable in weakly alkaline solutions. To determine the identity of the degradation products, a solution of CAR (0.5 g) in aqueous 0.2 M triethylammonium bicarbonate buffer (100 ml), pH 8.0, was allowed to stand at room temperature for 24 hr. The solution was then evaporated to dryness under reduced pressure at < 30°. The residual white solid was redissolved in 50 ml of methanol, 4.0 g of silica gel was added, and the mixture was evaporated. After further evaporations from methanol (2 × 50 ml) and chloroform (2 × 50 ml), the free-flowing powder was transferred to a column (25 × 1.5 cm) of silica (40 g) that had

been made up in chloroform. The products were eluted with chloroform–methanol (6:1, v/v); 10-ml fractions were collected. The eluted compounds were monitored by TLC analyses with iodine vapor visualization. Three major products, designated A, B, and C, were formed. R_f values for the parent compound and products were: CAR 0.75; A, 0.53; B, 0.50; and C, 0.42. Fractions containing individual components were combined and evaporated. The residual white solids were recrystallized from ethanol/ethyl acetate and stored under vacuum over P_2O_5.

Identity and synthesis of CAR degradation products. The products were characterized by NMR, MS and X-ray crystallography. Product A was identified as *N*-(methylcarbamoyloxy)acetamide. This material was synthesized for use as a chromatographic standard from acetohydroxamic acid and methyl isocyanate as follows. A solution of methyl isocyanate (0.5 ml, 8.48 mmoles) in chloroform (5 ml) was added to a finely divided suspension of acetohydroxamic acid (0.64 g, 8.53 mmoles) in chloroform (10 ml). After being allowed to stand for 24 hr at room temperature, the reaction mixture was evaporated to dryness. The residual white solid was recystallized from ethanol/ethyl acetate to yield white prisms. The yield was 0.85 g (76%). The compound was spectrally and chromatographically identical to product A. ^{1}H NMR [$(CD_3)_2SO$]: δ 1.80 (s, 3H, CH_3CO), 2.60 (d, 3H, NHCH_3, J_{HH} = 5 Hz), 7.43 (br. s, 2H, N*H*CH$_3$ and O*H*). ^{1}H NMR (D_2O): δ 1.94 (s, 3H, CH_3CO), 2.67 (s, 3H, NHCH_3). MS, *m/e* 132 (M^+), 75 ($M^+ - CH_3NCO$).

Product B was identified as *N*-(methylcarbamoyloxy)-*N'*-methylurea. This compound arises from the hydrolytic cleavage of the N-acetyl group of CAR. ^{1}H NMR [$(CD_3)_2SO$]: δ 2.58 (d, 3H, NHCH_3, J_{HH} = 5 Hz), 2.55 (d, 3H, NHCH_3, J_{HH} = 5 Hz); 6.67 (q, 1H, N*H*CH$_3$, J_{HH} = 5 Hz), 7.27 (q, 1H, N*H*CH$_3$, J_{HH} = 5 Hz); 9.27 (s, 1H, N*H*O). ^{1}H NMR (D_2O): δ 2.71 (s, 3H, NHCH_3), 2.74 (s, 3H, NHCH_3). MS, *m/e* 90 ($M^+ - CH_3NCO$).

Product C was identified as *N*-hydroxy-*N'*-methylurea. It was synthesized from methylisocyanate and hydroxylamine as follows. A solution of methylisocyanate (0.5 ml, 8.48 mmoles) in chloroform (5 ml) was added to a suspension of hydroxylamine (0.28 g, 8.5 mmoles) in chloroform (5 ml). The reaction mixture was stirred overnight and then evaporated to dryness. The remaining white solid was recrystallized from ethanol. The yield was 0.48 g (63%). ^{1}H NMR [$(CH_3)_2SO$]: δ 2.60 (d, 3H, NHCH_3, J_{HH} = 5 Hz), 6.57 (q, 1H, N*H*CH$_3$, J_{HH} = 5 Hz), 8.17 (s, 1H, N*H*OH or NHO*H*), 8.45 (s, 1H, N*H*OH or NHO*H*). MS, *m/e* 90 (M^+).

Plasma sample preparation and chromatography. Analytical determinations of CAR in plasma were done with a Waters Associates model 204 chromatograph equipped with a μBondapak C_{18} reverse phase column (30 cm × 4.0 mm i.d.). Plasma was extracted twice with equal volumes of chloroform. CAR extraction efficiency averaged 90% or more in individual experiments. The combined organic phases were evaporated to dryness and then reconstituted with 300 μl of water. A 200-μl aliquot was applied to the HPLC to determine the parent CAR compound. The aqueous layers were filtered through Amicon Centriflo membrane cones (type F_{25}) to remove plasma proteins. The filtrates were injected onto the HPLC to determine the ^{14}C-water-soluble product (WSP).

Samples were eluted isocratically at a flow rate of 1.0 ml/min with methanol–water (1:9, v/v) as mobile phase. Drug was detected with an ultraviolet monitor set at 210 nm. The location of unchanged CAR in biological fluid samples was ascertained by co-chromatographic elution profiles using a freshly prepared CAR standard. Under these conditions, CAR eluted from the HLPC column with a retention time of 8 min. Eluent was collected at 1-min intervals and mixed with 11 ml of scintillation fluid. Recovery of radioactively labeled CAR from the HLPC column was 85% or more.

Determination of $^{14}CO_2$ and ^{14}C-water-soluble product. The formation of $^{14}CO_2$ from [*acetyl*-1-^{14}C]CAR in the presence of rat brain homogenate was determined. Male Sprague–Dawley rats (200–300 g) were killed by decapitation, and a 50% brain homogenate (w/v) in cold 150 mM Tris buffer (pH 7.7) was prepared and centrifuged at 8000 *g* for 20 min to remove large particulate material. The protein content of the brain homogenate was determined by the method of Bradford [3]. A final concentration of 1 mg/ml of brain homogenate protein was used in each incubation.

$^{14}CO_2$ formation was determined by a modification of the procedure of Lapointe and Cohen [4]. Glass fiber filter disks wetted with a 7% (w/v) barium hydroxide solution were placed on the tops of plastic 24-well tissue culture chambers. Aliquots of homogenates, [^{14}C]CAR, and physostigmine solutions were added directly to the chambers which were then incubated at 37° for fixed time periods. A positive control consisting of brain homogenate, [^{14}C]ornithine, and ornithine decarboxylase was included in each experiment to ensure reproducibility of $^{14}CO_2$ trapping.

Degradation of CAR in the presence of human plasma and acetylcholinesterase. [^{3}H]CAR was incubated for 1 hr at 37° at a concentration of 10^{-4} M with potassium phosphate buffer (pH 7.4), plasma that was heat-deactivated at 100° for 30 min, or a solution of acetylcholinesterase (AChase) (3 molar unit excess) in 0.05 M potassium phosphate buffer, pH 7.4. The incubates were then diluted with 6 vol. of methanol and agitated for 30 sec. After centrifugation (5000 *g*, 10 min) to remove precipitated protein, the supernatant fractions were aspirated and concentrated to dryness under a stream of nitrogen. The residues were reconstituted in a 0.2-ml solution of methanol containing 0.5 mg of each of the following unlabeled carrier compounds: CAR and compounds A, B, and C. The reconstituted mixtures were applied to TLC analytical plates (20 × 20 cm), which were developed in chloroform–methanol (6:1, v/v). Areas corresponding to CAR and compounds A, B, and C were scraped off and transferred to liquid scintillation counting vials. One milliliter of water was added to elute the products, and the contents of the vials were mixed on a Vortex apparatus for 30 sec. Counting fluid (PCSA, Amersham/Searle, Chicago, IL) was added, and,

after allowing the samples to dark adapt for 24 hr, their radioactivity was determined.

Enzymatic assays. AChase activity was measured by the method of Wilson *et al.* [5]. In brief, a homogenate of N1E-115 neuroblastoma cells was prepared by sonication in 50 mM Tris/0.2% Triton buffer at pH 7.4. An aliquot was preincubated for 5 min with various concentrations of CAR before the assay was started by the addition of [^{14}C]acetylcholine iodide in 100 mM sodium phosphate buffer (pH 7.1). The reactions were stopped by the addition of 100 μl of a 50 mg/ml solution of tetraphenylboron in 3-heptanone, which extracted the substrate. Radioactivity was determined in an aliquot of the aqueous phase containing the product.

Ribonucleotide reductase was prepared from Novikoff ascites tumor cells as previously described [6]. Inhibition of partially purified ribonucleotide reductase activity by CAR or CAR degradation product was determined as described [7, 8], except that [^{14}C]CDP was used as a substrate, and ADP and nucleoside diphosphate kinase were omitted.

In vitro-*cell resistance to CAR.* Two Chinese hamster ovary cell lines, NCR-30A0.7 and NCR-30A2, reported [9, 10] to be resistant to hydroxyurea and carbamoyloxyurea and to have increased ribonucleotide reductase activity were used along with a parental wild-type (WT) cell line to determine the extent of cross-resistance with CAR. The cell lines were maintained in MEM (Irvine Scientific, Irvine, CA) plus 10% fetal bovine serum. Cells grown in 60-mm tissue culture dishes were exposed to various CAR concentrations for 1 hr; the cultures were washed and grown in drug-free medium for 7 days before fixation in 10% formaldehyde and staining with 0.1% crystal violet.

The parental WT cell line was also used to determine the cytotoxicity of CAR degradation products A, B, and C. Cells were exposed to CAR or a degradation product for 1 hr and then washed and treated as described above.

Centrifugal elutriation. CHO cells were synchronized by centrifugal elutriation as described [11–13]. Approximately 1×10^8 cells in 20 ml of medium were introduced into the elutriation chamber at a flow rate of 12 ml/min and elutriated at room temperature by increasing the flow rate of medium through the chamber, stepwise, from about 12 to 30 ml/min. Twelve 50-ml fractions were collected. The centrifuge (model J-21C fitted with a model JE-6 elutriator rotor; Beckman Instruments, Palo Alto, CA) was operated at a constant rotor speed of 1700 rpm. Aliquots were withdrawn for cell counts and Coulter volume determinations, and another aliquot was fixed in 1 ml of saline and 3 ml of 70% ethanol for flow-cytometric analysis.

Following separation, known numbers of cells were plated into 60-mm culture dishes and allowed to attach for 45 min. The dishes were then treated for 1 hr with CAR in complete medium. The medium was then removed, the cells were rinsed once, and fresh medium was added. The dishes were incubated for 7–8 days to allow colony formation.

RESULTS

Degradation of [^{14}C]-CAR. Phase 1 clinical trials of CAR prompted us to determine the stability of this new antitumor agent in the presence of plasma. As seen in Fig. 2, the incubation of [1-^{14}C]CAR with human plasma resulted in a time- and temperature-dependent degradation. At 37°, for example, less than 33% of unchanged CAR was found remaining in the incubation medium at the end of 1 hr. In contrast, at 4°, about 70% of unchanged CAR was found.

Since clinical trials had demonstrated a marked propensity of this compound to be toxic to the central nervous system, we determined the degradation of CAR in the presence of a rat brain homogenate. A small percentage of [1-^{14}C]CAR was degraded to $^{14}CO_2$, indicating loss of the radioactive acetyl group (Fig. 3). Addition of physostigmine to the incubation medium reduced $^{14}CO_2$ formation by more than 50%, suggesting that such esterases as AChase might play a role in CAR metabolism. In addition to $^{14}CO_2$, unidentified ^{14}C-labeled products that were not extractable by chloroform were formed; over 60 min, they accounted for more than 70% of the all labeled incubation products. Adding physostigmine had little effect on the formation of these unidentified CAR metabolites (Fig. 4).

AChase inhibition. A direct interaction of CAR with AChase activity was examined using a neuro-

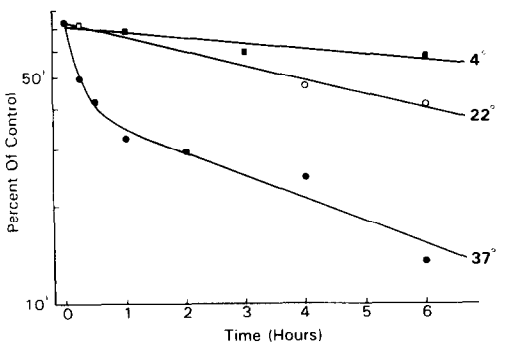

Fig. 2. Time- and temperature-dependent degradation of caracemide (10^{-4} M).

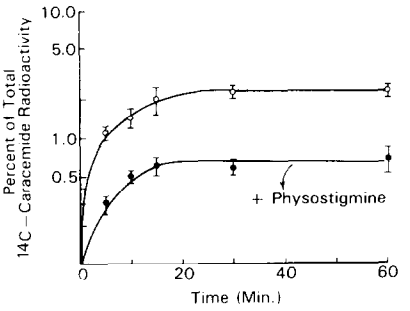

Fig. 3. Generation of $^{14}CO_2$ from [*acetyl*-1-^{14}C]CAR in the presence of rat brain homogenate (1 mg protein/ml; 37°). Key: (○) without physostigmine, and (●) in the presence of 10^{-3} M physostigmine. Data are presented as mean ± S.D. from three experiments.

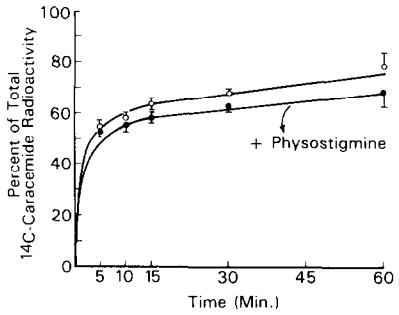

Fig. 4. Generation of ^{14}C-water-soluble product(s) from [*acetyl*-1-^{14}C]CAR rat brain homogenate (37°). Key: (○) without physostigmine, and (●) in the presence of 10^{-3} M physostigmine. Data are presented as mean ± S.D. from the three experiments.

Table 1. Identification of caracemide degradation products formed by incubation with human plasma, acetylcholinesterase, or phosphate buffer

Incubation condition	Percentages of total radioactivity applied to TLC plate			
	CAR	A	B	C
Buffer	25.3	28.8	19.2	8.1
Boiled human plasma	26.8	34.7	19.1	6.0
Human plasma	23.2	8.9	11.6	33.9
Buffer and acetylcholinesterase	20.6	12.1	11.5	5.5

[^{3}H]CAR was incubated with phosphate buffer (0.05 M, pH 7.4), human plasma, boiled human plasma, or phosphate buffer and acetylcholinesterase at 37° for 1 hr. Aliquots of the incubation mixtures were applied along with nonradioactive standards as carriers to thin-layer silica gel chromatography plates and developed as described in Materials and Methods.

blastoma cell line. As shown in Fig. 5, CAR was found to be a potent inhibitor of AChase activity in murine neuroblastoma cells (clone N1E-115) with an IC_{50} of 14 μM. The K_i for CAR in this *in vitro* system was found to be 8 μM as calculated from Dixon-plot analyses (data not shown).

Identification of [^{3}H]-CAR degradation products. Because the *N*-acetyl group in CAR was shown, in preliminary experiments, to be labile, [1-^{14}C-*acetyl*]-CAR was deemed inappropriate for determining the identity of all of the CAR degradation products. *N* - Acetyl - *N* - [^{3}H - *methylcarbamoyloxy*] - *N'* - methylurea ([^{3}H-*methyl*]CAR) was, therefore, synthesized in our laboratory. Incubation of [^{3}H-*methyl*]CAR in an alkaline buffer solution produced three major degradation products. Independent syntheses of presumed degradation products and structural analyses by NMR, mass spectrometry, X-ray crystallography, and HPLC permitted the assignments depicted in Fig. 1.

Incubation of [^{3}H-*methyl*]CAR with either phosphate buffer or boiled (denatured) plasma with subsequent TLC analyses demonstrated that the principal CAR degradation compound was product A: *N*-(methylcarbamoyloxy)acetamide. As the results (Table 1) show, addition of human plasma to [^{3}H-*methyl*]CAR yielded principally product C and other chloroform nonextractable products that remained at the origin of the TLC plate.

CAR mechanism of action. CAR has been reported to inhibit ribonucleotide reductase but only at a concentration nine times that of hydroxyurea (HU) to produce a similar level of enzyme inhibition [8]. In our study, CAR was again shown to inhibit ribonucleotide reductase activity by 50% at 0.9 mM, an approximately 8-fold higher concentration than the IC_{50} of HU in this *in vitro* assay system. Degradation product A (2 mM) inhibited enzyme activity by 35%; it was about one-fourth as active as CAR. Product B (2 mM) inhibited enzyme activity by less than 10%. Product C (*N*-hydroxy-*N'*-methylurea), however, was more active than CAR, with an IC_{50} of 0.17 mM, only 55% higher than that of HU. Since

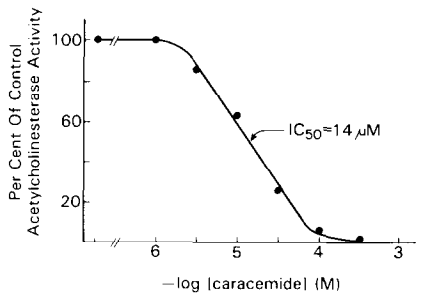

Fig. 5. Inhibition of N1E-115 murine neuroblastoma acetylcholinesterase by CAR. Various CAR concentrations were preincubated in duplicate with a homogenate of N1E-115 cells before beginning the assay for acetylcholinesterase by adding [^{14}C]acetylcholine. After another 5 min of a further incubation, the reaction was stopped and the product isolated. One hundred percent is equivalent to an activity of 28.5 μmoles/min/10^6 cells. The IC_{50} for CAR was 14 μM.

Table 2. Effect of caracemide on two hydroxyurea-resistant Chinese hamster ovary cell lines

Drug	Concn (mM)	Plating efficiency (%) WT	NCR-30A0.7	NCR-30A2
Control		91.7	86.2	85.0
Hydroxyurea	1.0	0.1	6.2	7.5
Caracemide	0.05	55.5	51.0	53.7
	0.11	33.4	36.2	41.8
	0.21	22.9	27.9	28.0
	0.79	<0.2	<0.2	<0.2

Cells were exposed to caracemide or hydroxyurea for 1 hr as described in Materials and Methods. Colonies were counted and plating efficiency was determined after 72-hr incubation in drug-free medium. The data represent the results of a typical experiment repeated twice with similar findings.

CAR can be regarded as a derivative of *N*-carbamoyloxyurea, we chose to compare the activities of CAR and HU with those of two Chinese hamster ovary cell lines selected for resistance to *N*-carbamoyloxyurea (Table 2). Although HU demonstrated a significant antiproliferative effect against the parental wild-type cell line, both *N*-carbamoyloxyurea-resistant lines were also resistant to this drug, as has been reported [10]. CAR, however, was equally effective against both the parental wild type and the resistant cell lines.

Further comparisons of CAR and HU mechanisms of action showed, first, that CAR killed cells over several log dosages whereas HU, even at high doses, killed only about 50% of the cells in an asynchronous growing population (data not shown). This suggested that HU was specific for S-phase cells, as has been reported [12], and that CAR killed cells in all phases of the cell cycle. Experiments with CHO cells synchronized in different phases of the cell cycle confirmed this interpretation. Thus, the results presented in Fig. 6 show that CAR killed cells in all phases of the cell cycle, with only a slight preference for killing cells in late S : G2 phase, and that HU kills only S-phase cells.

In additional experiments to ascertain the relative cytotoxicity of the CAR degradation products, we found that, on an equimolar basis, compound A was nearly as cytotoxic to CHO cells as was the parent compound. Compounds B and C were not cytotoxic in the same range of concentrations (data not shown).

DISCUSSION

In these studies CAR has been demonstrated to be chemically unstable and degraded not only in the presence of plasma but also in buffers at physiologic pH. The principal degradation product in the presence of phosphate buffer was *N*-(methylcarbamoyloxy)acetamide. This compound was the only degradation product found to have significant cytotoxicity (data not shown). It was less than one-third as potent as CAR, however, in its ability to kill cells. Human plasma changed the degradation of CAR such that the weakly cytotoxic compound *N*-hydroxy-*N'*-methylurea (product C) was produced. The formation of radiolabeled C from *N*-acetyl-*N*-[^{3}H-*methylcarbamoyloxy*]-*N'*-methylurea is, at first sight, anomalous. However, further degradation of compound A or B by cleavage of the *N*-acetyl and *N*-(methylcarbamoyl) groups, respectively, would generate *O*-(methylcarbamoyl)hydroxylamine [i.e. $H_2NOC(O)NHCH_3$], a thermodynamically unstable product, which would be expected to undergo facile intramolecular rearrangement to give *N*-methyl-*N'*-hydroxyurea. The precise mechanism of formation of this compound, however, is unknown.

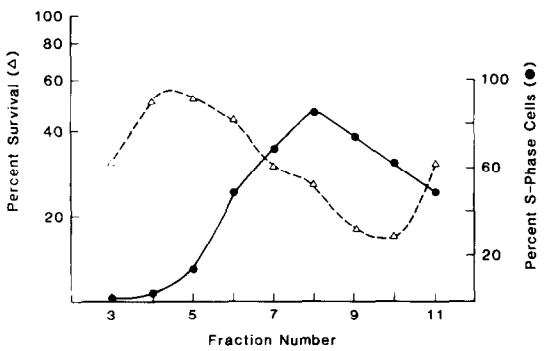

Fig. 6. Cell-cycle survival pattern for CAR. CHO cells were separated by centrifugal elutriation into fractions numbered 3 to 11. Cells from each fraction were plated and treated for 1 hr with 10 μg/ml CAR in complete medium. The proportion of cells in S-phase as determined by flow cytometry is shown for each fraction.

The biotransformation of CAR to products A, B, and C was most likely mediated by various plasma amidases and esterases. Even the relatively selective enzyme AChase produced a small increase in the release of $^{14}CO_2$ from the 1-^{14}C-acetyl position of [^{14}C]CAR. This enzyme-mediated degradation was partially inhibited by physostigmine. It is not known, however, whether other more general esterase and amidase inhibitors would be more specific in altering the extent or pattern of CAR degradation.

CAR, an *N*-methylurea derivative, has certain structural similarities to HU. Our current studies suggest, however, that the mechanism of cytotoxicity of CAR and HU have little in common. Like HU, CAR inhibited ribonucleotide reductase, but at about 10-fold higher levels than those required for inhibition by HU. The general conclusion has been that ribonucleotide reductase inhibition by HU-like compounds requires a free C-NOH group [14]. Although CAR lacks such a chemical group, the principal degradation product C formed in human plasma contains this group and is an effective inhibitor. Hence, it is possible that CAR degradation products may contribute substantially to the observed inhibition of ribonucleotide reductase. Since CAR may also be considered a derivative of *N*-carbamoyloxyurea, we examined the activity of CAR against two CHO cell lines with different levels of resistance to *N*-carbamoyloxyurea. Whereas these cell lines demonstrated the previously observed cross-resistance to HU, consistent with increased intracellular levels of ribonucleotide reductase, CAR was equally effective against parental and resistant cells, suggesting a different mechanism of cytotoxic action that does not depend on the relative level of ribonucleotide reductase activity. Finally, HU is considered to be a classical S-phase specific agent, whereas CAR was found to be non-cell-cycle phase-specific in its cell-killing ability. Thus, although CAR structurally resembles HU, it appears to have a different mechanism or mechanisms of cytotoxic action.

A preclinical toxicologic evaluation of CAR in mice and beagle dogs produced evidence of a generalized hypercholinergic response [1]. A recent report of a Phase I clinical trial of CAR also noted serious CAR-mediated neurotoxicity in the form of confusion, disorientation, and agitation [2]. In this study, we demonstrated the ability of CAR to inhibit AChase activity associated with neuroblastoma cells. This inhibition may be responsible, in part, for the cholinergic stimulation and toxicity observed in experimental animals and humans.

When caracemide is degraded to product A, methyl isocyanate (MIC) is also produced. The *in vivo* production of methyl isocyanate may, in fact, account for some of the observed pharmacologic and toxicologic effects of CAR itself. Isocyanates are extremely reactive chemicals resembling α,β-unsaturated aldehydes and ketones in their propensity to undergo addition reactions with a variety of cellular products containing active hydrogen atoms. The knowledge of methyl isocyanate toxicology, however, is limited no doubt due to the volatile and flammable nature of the compound.

MIC has been shown to bind to and inactivate acetylcholinesterase and is suspected of causing permanent damage to the nervous system [15]. Because of its chemical nature, MIC would be expected to bind avidly to critical target macromolecules in all phases of the cell cycle and demonstrate no cell cycle specificity with regard to cell killing. This is similar to the action of CAR. Likewise, if MIC contributes significantly to the cytotoxicity of CAR, it would not be expected to demonstrate cross-resistance with HU resistant cell lines. Product A was found in the present study to be more cytotoxic than either product B or C; it is of interest that MIC is readily obtained from product A but not from B or C. Finally, MIC itself will spontaneously react with water to yield methylamine and carbon dioxide. Some of the CO_2 production observed with CAR degradation may, in fact, be due to MIC degradation. The precise role of MIC with regard to CAR antitumor efficacy and toxicology is, as yet, undetermined but may be of importance with respect to ongoing clinical trials of caracemide.

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