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Substituted 4-hydroxyproline di- and tri-peptides as cytotoxic agents

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Summary. 4-Hydroxyproline di- and tri-peptides and N-cbz-hydroxypropyl-glycinamides were observed to be potent cytotoxic agents against the growth of suspended single cells, L-1210, $Tmolt_3$, and HeLa-S^3. The agents were not as potent against the growth of cultured solid tumor cells. Selected derivatives were investigated for their mode of action in $Tmolt_3$ leukemia cells. The compounds selectively inhibited DNA synthesis at 50 and 100μM. The target site of action of the agents appeared to be the purine *de novo* pathway with marked inhibition of the activities of the two regulatory enzymes of the pathway, i.e. PRPP amido-transferase and IMP dehydrogenase. d[NTP] pools were reduced by the agents consistent with their overall reduction of DNA synthesis. Other marginally inhibited targets of the agents were r-RNA polymerase and TMP-kinase activities. The DNA molecule itself did not appear to be a target of these agents.

Keywords: Amino acids – Anti-neoplastic amino acids – Purine and DNA synthesis inhibitors – PRPP amido transferase and IMP dehydrogenase inhibitors

Introduction

Carbobenzoxy[cbz]phenylalanine vinyl esters and 1,2-dibromoethyl esters have previously been shown to be potent anti-neoplastic and anti-inflammatory agents in rodents (Loeffler et al., 1977a). These studies were extended to other amino acids, e.g. leucine, glycine, glutamic acid, aspartic acid and tyrosine which afforded similar activities (Loeffler et al., 1977b). Recently a series of N-cbz-hydroxyprolylglycinamides and related derivatives were synthesized and tested as anti-fibrotic agents, i.e. for the inhibition of prolyl-4-hydroxylase activity, an enzyme required in collagen synthesis (Canne, 1993). These compounds were designed as antimetabolites of hydroxyproline. Due to the similarity in structure to known peptide anti-neoplastic agents, e.g. boron peptides, it was decided to test these new

agents for cytotoxic activity in murine and human cultured cells (Hall et al., 1993).

Materials and methods

Source of compounds

The N-cbz-4-hydroxyprolylglycinamide derivatives (Fig. 1) were synthesized as reported (Canne, 1993). All radioisotopes were purchased from New England Nuclear (Boston, MA) unless otherwise indicated. Radioactivity was determined in Fisher Scintiverse scintillation fluid with correction for quenching. Substrates and cofactors were obtained from Sigma Chemical Co. (St. Louis, MO).

Pharmacological methods

Cytotoxicity

The drugs were placed in solution by homogenization in 0.05% Tween 80/H_2O; the solution was a stable stock at 1 mM concentration of drug. The vehicle, 0.05% Tween 80/H_2O, functioned as the control for all biochemical experiments. Compounds **1–9** (Table 1) were tested for cytotoxic activity. These solutions of drugs and standards were sterilized by passing them through an acrodisc (0.45 μm). The following cell lines were maintained by literature techniques (Geran, et al., 1972): murine L_{1210} lymphoid leukemia, human $Tmolt_3$ acute lymphoblastic T cell leukemia, colorectal adenocarcinoma SW480, lung bronchogenic MB-9812, osteosarcoma TE418, KB epidermoid nasopharynx, HeLa-S^3 suspended cervical carcinoma, and glioma EH 118 MG. The NCI protocol was used to assess the cytotoxicity of the test compounds and standards in each cell line during exponential log growth. Values for cytotoxicity were expressed as ED_{50} [μg/ml], i.e. the concentration of the compound inhibiting 50% of cell growth. ED_{50} values were determined by the trypan blue exclusion technique (Geran et al., 1972) after 3 days (~3 doubling times). A value of less than 4 μg/ml was required for significant activity of growth inhibition according to the NCI protocol. Solid tumor cytotoxicity was determined utilizing crystal violet/MeOH and read at 580 nm (Molecular Devices) after convergence of the control, ~4–6 days (Leibovitz et al., 1976).

Anti-neoplastic activity

All of the compounds were evaluated for their ability to reduce Ehrlich ascites carcinoma growth in CF_1 male mice (28 ± 3 g) (Hall et al., 1978). Mice were implanted with 2×10^6 tumor cells I.P. on day zero and randomly sorted into groups of six. Test drugs were administered at 8 mg/kg/day I.P. on days 1–9. 6-Mercaptopurine (6-MP) at 1 mg/kg/day and 5-fluorouracil (5FU) at 27.5 mg/kg/day were used as standards in the screen. On day 10 the tumor was harvested and the volume and astrocrit determined from which the percent inhibition of tumor growth was determined (Hall et al., 1978).

Incorporation studies

The effects of the agents over a concentration range of 25, 50 and 100 μM on DNA, RNA and protein syntheses was determined by the incorporation of labeled precursors ^{3}H-thymidine, ^{3}H-uridine and ^{3}H-leucine, respectively into 10^6 $Tmolt_3$ leukemia cells for 60 min (Laio et al., 1976). The incorporation of ^{14}C-glycine (53.0 mCi/mmol) into purines was obtained (Cadman et al., 1981). The incorporation of ^{14}C-formate (53.0 mCi/mmol) into pyrimidines was determined in a similar manner (Christopherson et al., 1981). $Tmolt_3$ leukemic cells were in the log growth phase with a doubling time of ~26 hours which were used for all of the biochemical experiments.

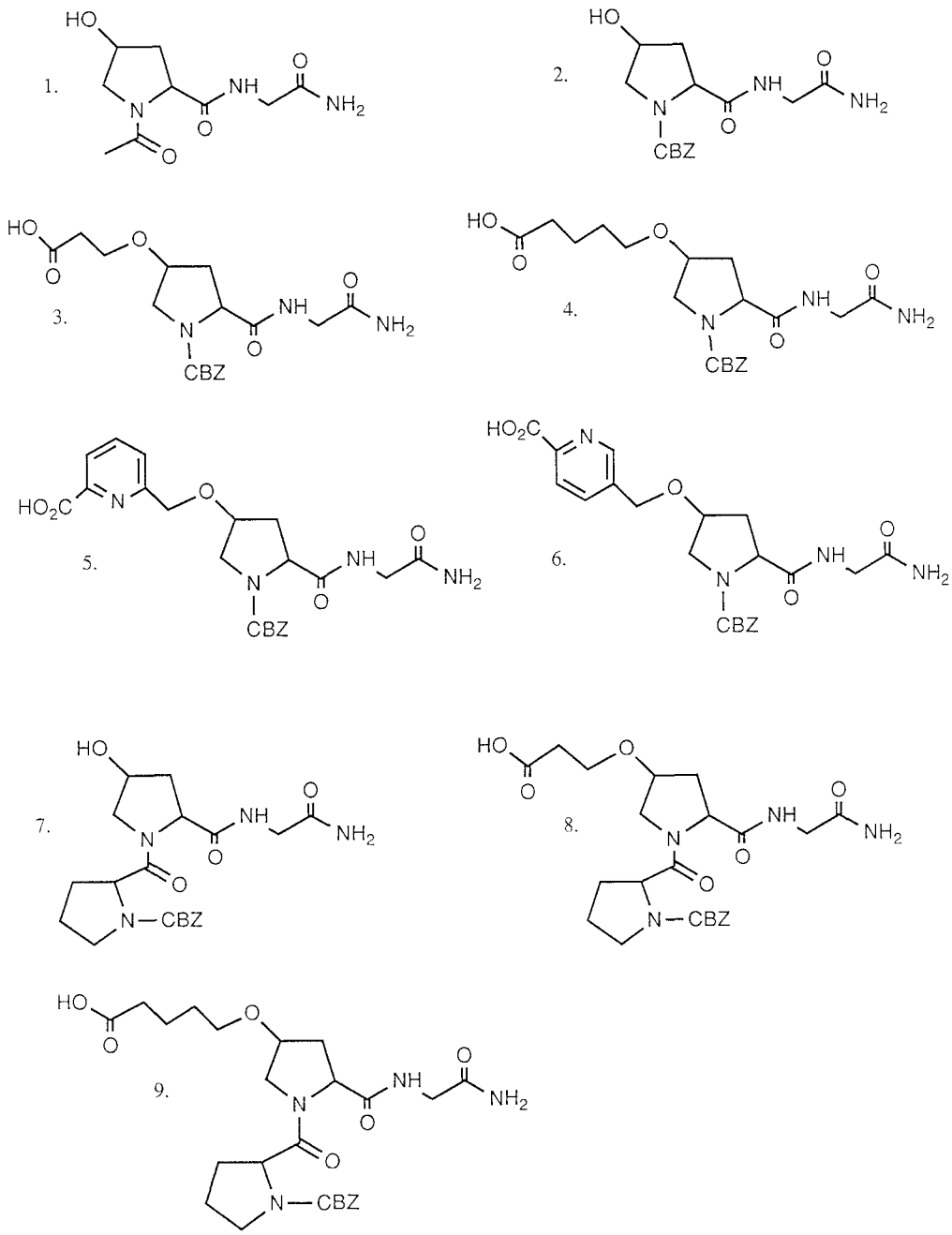

Fig. 1. Structures of substituted 4-hydroxyproline di & tripeptides

Enzyme assays

Inhibition of various enzyme activities was performed by first preparing the appropriate $Tmolt_3$ cell homogenates or subcellular fractions, then adding the drug to be tested during the enzyme assay. All of the conditions of the enzyme assays were optimized for $Tmolt_3$ cells so that the reaction was linear over the 60 min. For the concentration response studies, inhibition of enzyme activity was determined at 25, 50 and 100 μM of compounds

Table 1. Cytotoxicity of N-Cbz-4-hydroxyprolylglycinamide and related derivatives

ED_{50} values [μg/ml]
N = 4

Cp'd #	L-1210	$Tmolt_3$	HeLa-S^3		Adenocarcinoma		KB Naso-pharynx	Skin	Lung		Osteo bone sarcoma	Glioma
	Leukemia		Uterine	Solid	Colon SW-480	Ileum HCT-8		A431	A549	M9812		Brain
1	1.70	2.04	1.69	5.42	9.40	7.11	5.92	6.47	6.47	6.42	2.92	8.56
2	3.77	0.89	1.09	7.46	8.28	11.52	10.61	10.18	6.79	7.55	7.87	8.03
3	2.30	0.78	2.09	8.39	6.09	11.35	10.74	9.27	6.38	8.12	7.67	8.53
4	2.46	0.90	1.70	8.50	8.37	10.83	11.91	8.52	7.91	8.74	7.58	9.50
5	3.52	2.52	2.01	9.21	8.58	8.67	11.93	8.61	8.40	8.56	7.03	8.22
6	3.36	1.07	1.96	9.48	8.47	9.15	13.59	8.28	6.81	8.18	6.69	7.29
7	3.81	1.97	1.74	8.03	6.84	8.62	6.93	6.92	5.39	8.45	6.88	6.68
8	4.32	0.52	1.96	8.83	8.36	8.23	10.26	7.06	5.92	8.95	7.27	7.27
9	2.62	0.93	2.75	8.06	8.74	11.16	7.77	8.82	6.64	8.51	8.48	8.48
5FU	1.41	2.14	2.47	4.11	3.09	1.12	1.25	0.61	3.58	5.64	3.52	1.28
Ara C	2.76	2.67	2.13	4.74	3.42	2.54	2.84	0.92	4.69	6.16	0.86	1.88
Hydroxyurea	2.67	3.18	1.96	8.12	4.74	1.77	5.29	3.21	7.37	7.18	2.87	2.57
6MP	2.42	1.62	2.12	5.61	3.61	1.15	11.04	3.42	4.71	4.29	9.39	4.46

5FU 5-fluorouracil; *Ara-C* cytosine arabinoside; *6MP* 6-mercaptopurine.

2, **3**, and **8** after 60 min incubations. DNA polymerase α activity was determined in cytoplasmic isolated extracts (Eichler et al., 1977). Nuclear DNA polymerase β was determined by isolating nuclei (Mamaril et al., 1970). The DNA polymerase assay for both α and β was determined with ^{3}H-thymidine-5′-triphosphate [TTP] (Sawada et al., 1974). Messenger-, ribosomal- and transfer-RNA polymerase enzymes were isolated with different concentrations of ammonium sulfate; and individual RNA polymerase activities were determined using ^{3}H-uridine-5′-triphosphate [UTP] (Anderson et al., 1975; Hall et al., 1974). Ribonucleoside reductase activity was measured using ^{14}C-cytidine-5′-diphosphate [CDP] with dithioerythritol (Moore and Hurlbert, 1966). The deoxyribonucleotides ^{14}C-deoxyribocytosine-5′-diphosphate [dCDP] were separated from the ribonucleotide [CDP] by TLC on polyethyleneimine cellulose [PEI] plates. Thymidine, thymidine-5′-monophosphate [TMP] and thymidine-5′-diphosphate [TDP] kinase activities were determined using ^{3}H-thymidine (58.3 mCi/mmol) (Maley and Ochoa, 1958). Carbamyl phosphate synthetase activity was determined (Kalman et al., 1966). The product, citrulline, was quanitated colorimetrically (Archibald, 1944). Aspartate transcarbamylase activity was measured (Kalman et al., 1966). The product, carbamyl aspartate, was quanitated colorimetrically (Koritz and Cohen, 1954). Thymidylate synthetase activity was analyzed by the $^{3}H_2O$ released which was proportional to the amount of TMP formed from ^{3}H-2′-deoxyribouridine-5′-uridine monophosphate(dUMP) (Kampf et al., 1976). Dihydrofolate reductase activity was determined by a spectrophotometric method (Ho et al., 1971). PRPP-amidotransferase activity was determined by the method of Spassova et al. (1976). Inosine-5′-monophosphate [IMP] dehydrogenase activity was analyzed with 8-^{14}C-IMP (54 mCi/mmol) (Amersham, Arlington Heights, IL) after separating xanthosine-5′-monophosphate [XMP] on PEI plates (Fisher Scientific) by TLC (Becker and Lohr, 1979). Protein content was determined for the enzymatic assays by the Lowry technique (Lowry et al., 1951).

DNA studies

$Tmolt_3$ deoxyribonucleoside triphosphates [dNTP] were extracted (Bagnara and Finch, 1971). d[NTP] levels were determined with calf thymus DNA [ct-DNA], *E. coli* DNA polymerase I, non-limiting amounts of the three deoxyribonucleoside triphosphates not being assayed, and either 0.4 mCi of (^{3}H-methyl)-dTTP or (5-^{3}H)-dCTP (Hunting and Henderson, 1982). The effects of compounds **2**, **3**, and **8** on DNA strand scission was determined by the methods of Suzuki et al. (1978), Pera et al. (1981) and Woynarowski et al. (1981). $Tmolt_3$ leukemia cells were incubated with 10 μCi thymidine methyl-^{3}H, 84.0 Ci/mmol for 24 hr at 37°C. $Tmolt_3$ cells (10^7) were harvested and then centrifuged at 600 g × 10 min in phosphate saline buffer [PBS]. They were later washed and suspended in 1 ml of PBS. Lysis buffer (0.5 ml; 0.5 M NaOH, 0.02 M EDTA, 0.01% Triton X-100 and 2.5% sucrose) was layered onto a 5–20% alkaline-sucrose gradient (5 ml; 0.3 M NaOH, 0.7 KCl and 0.01 M EDTA); this was followed by 0.2 ml of the cell preparation. After the gradient was incubated for 2.5 hr at room temperature, it was centrifuged at 12,000 RPM at 20°C for 60 min (Beckman rotor SW60). Fractions (0.2 ml) were collected from the bottom of the gradient, neutralized with 0.2 ml of 0.3 N HCl, and measured for radioactivity. Thermal calf thymus DNA denaturation studies, DNA u.v. absorption studies and DNA viscosity studies were conducted after incubation of compounds **2**, **3**, and **8** at 100 μM at 37°C for 24 hr (Zhao et al., 1987).

Statistical analysis

Data is displayed in Table 2 as the means ± standard deviations of the mean. N is the number of samples or animals per group. The Student's "t"-test was used to determine the probable level of significance (p) between test samples and control samples.

Table 2. Effects of compounds **2**, **3**, and **8** on Tmolt$_3$ leukemia cell metabolism over 60 min

Percent of control (X + SD) (N = 6)

Assay	Control	Compound **2**			Compound **3**			Compound **8**		
		25 μM	50 μM	100 μM	25 μM	50 μM	100 μM	25 μM	50 μM	100 μM
DNA synthesis	100 ± 6a	74 ± 5*	71 ± 5*	48 ± 4*	65 ± 4*	59 ± 5*	49 ± 4*	150 ± 6*	43 ± 4*	44 ± 4*
RNA synthesis	100 ± 5b	89 ± 5	50 ± 4*	25 ± 3*	124 ± 6	135 ± 6*	158 ± 6*	126 ± 5*	136 ± 6*	151 ± 7*
Protein synthesis	100 ± 7c	88 ± 5	114 ± 6	130 ± 6*	158 ± 7*	175 ± 6*	210 ± 7*	180 ± 6*	81 ± 6	77 ± 5
DNA polymerase alpha	100 ± 6d	106 ± 5	75 ± 3*	70 ± 4*	105 ± 6	95 ± 6	90 ± 5	99 ± 5	86 ± 6	81 ± 5*
mRNA polymerase	100 ± 6e	97 ± 5	84 ± 4	82 ± 6	120 ± 7	106 ± 5	91 ± 6	87 ± 5	82 ± 4*	78 ± 4*
rRNA polymerase	100 ± 6f	58 ± 4*	53 ± 3*	50 ± 3*	66 ± 5*	54 ± 4*	54 ± 3*	65 ± 5*	64 ± 4*	62 ± 4*
tRNA polymerase	100 ± 5g	103 ± 6	97 ± 6	90 ± 6	100 ± 7	95 ± 5	94 ± 6	104 ± 6	98 ± 5	96 ± 6
Ribonucleoside reductase	100 ± 8h	105 ± 6	82 ± 3	86 ± 5	92 ± 5	84 ± 6	84 ± 5	95 ± 6	96 ± 5	94 ± 5
Dihydrofolate reductase	100 ± 7i	130 ± 5*	106 ± 5	105 ± 5	121 ± 6	106 ± 5	98 ± 6	110 ± 6	105 ± 4	80 ± 4*
Purine de novo synthesis	100 ± 8j	44 ± 4*	40 ± 3*	38 ± 2*	52 ± 4*	38 ± 3*	36 ± 3*	26 ± 3*	21 ± 2*	21 ± 3*
PRPP amido transferase	100 ± 6k	26 ± 3*	24 ± 2*	21 ± 2*	98 ± 5	94 ± 4	42 ± 4*	95 ± 5	68 ± 5*	48 ± 4*
IMP dehydrogenase	100 ± 7l	106 ± 5	85 ± 4	79 ± 5*	82 ± 5	65 ± 4*	47 ± 4*	86 ± 5	54 ± 5*	48 ± 3*
Pyrimidine de novo synthesis	100 ± 6m	99 ± 7	98 ± 6	96 ± 6	104 ± 6	103 ± 5	93 ± 5	98 ± 7	96 ± 5	97 ± 5
Carbamyl phosphate synthetase	100 ± 7n	94 ± 4	92 ± 5	91 ± 5	108 ± 7	100 ± 6	81 ± 7	85 ± 5	81 ± 4*	78 ± 4*
Aspartate transcarbamylase	100 ± 8o	99 ± 6	95 ± 5	86 ± 5	97 ± 6	95 ± 5	93 ± 4	99 ± 6	92 ± 4	91 ± 7
Thymidylate synthetase	100 ± 5p	120 ± 6	125 ± 5	143 ± 6*	109 ± 7	134 ± 6	147 ± 7*	102 ± 6	108 ± 6	120 ± 6
Thymidine kinase	100 ± 6q	96 ± 5	88 ± 4	91 ± 5	99 ± 7	102 ± 6	104 ± 6	107 ± 5	92 ± 6	81 ± 5
Thymidine monophosphate kinase	100 ± 5r	105 ± 5	78 ± 3	77 ± 3*	103 ± 7	88 ± 5	90 ± 6	103 ± 6	90 ± 5	79 ± 4*
Thymidine diphosphate kinase	100 ± 5s	92 ± 3	89 ± 5	81 ± 5*	84 ± 6	101 ± 6	101 ± 6	83 ± 6	85 ± 5	104 ± 8
d(ATP)	100 ± 6t	–	–	67 ± 4*	–	–	79 ± 5*	–	–	63 ± 5*
d(GTP)	100 ± 5u	–	–	49 ± 4*	–	–	54 ± 4*	–	–	54 ± 3*
d(CTP)	100 ± 6v	–	–	64 ± 3*	–	–	59 ± 2*	–	–	61 ± 3*
d(TTP)	100 ± 6w	–	–	133 ± 6*	–	–	121 ± 5	–	–	182 ± 7*

Control values for 10^6 cells/hr

a 7,719 dpm
b 1,014 dpm
c 17,492 dpm
d 9,019 dpm
e 1,343 dpm
f 325 dpm
g 400 dpm
h 48,780 dpm
i 0.144 D O.D. units
j 28,624 dpm
k 0.0878 O.D. units
l 19,575 dpm. units
m 19,758 dpm
n 0.807 mol N-carbamyl aspartate
o 0.273 mmol citrulline
p 77,616 dpm
q 1,371 dpm
r 1,179 dpm
s 1,891 dpm
t 17.07 dpm
u 13.58 pmoles
v 33.60 pmoles
w 31.04 pmoles

* $P \leq 0.001$ Student's "t" test.

Results

In the *in vivo* Ehrlich ascites tumor model in CF_1 mice, compound **1** at 8 mg/kg/day afforded 100% inhibition of growth, compounds **3** and **5** afforded 97.5% inhibition, and compound **6** afforded 96% inhibition. 6MP at 1 mg/kg/day resulted in 99.9% inhibition of growth of Ehrlich ascites carcinoma. 5FU at 27.5 mg/kg resulted in 90% suppression of tumor growth. Compounds **2**, **4**, **7** and **9** caused 74%, 72%, 77.6% and 71% inhibition, respectively. Compound **8** was toxic at 8 mg/kg/day and resulted in the death of all of the animals (N = 6).

The N-cbz-4-hydroxyprolylglycinamides and related derivatives demonstrated good cytotoxic activity against the growth of murine and human cultured tumor cells. Significant activity was denoted as agents with ED_{50} values of less than 4 μg/ml according to the NCI protocol. Compounds **1–7** and **9** demonstrated potent cytotoxicity against the growth of L1210 lymphoid leukemia demonstrating activities in the range of the clinical standard agents. All nine compounds were active against $Tmolt_3$ growth. Selected compounds, **2**, **3**, **4**, **8**, and **9** afforded ED_{50} values $< 1\,\mu$g/ml, i.e. these agents were more potent than the clinical standards used in the assay. All of the compounds were active against HeLa-S[3] suspended uterine cell growth with ED_{50} values $< 3\,\mu$g/ml. The compounds were generally not active in the human colon SW480 or ileum adenocarcinoma HCT-8, KB nasopharynx, skin epidermoid A431, solid HeLa uterine, glioma or rat UMR-106 osteosarcoma screen. Compound **1** did result in an ED_{50} value of 2.93 μg/ml in the rat UMR-106 osteosarcoma screen and 5.42 μg/ml in the human solid HeLa uterine screen. Compounds **8** and **9** caused ED_{50} values of 5.39 and 5.92 μg/ml in the human lung A549 screen. As can be noted the N-cbz-4-hydroxyprolylglycinamides and related derivatives in the solid tumor screens were not as active as in the suspended cell lines, but many of the standards also demonstrated this same pattern of activity, i.e. afforded $ED_{50} > 4\,\mu$g/ml.

In the $Tmolt_3$ leukemia cultured cell line compounds **2**, **3** and **8** suppressed DNA synthesis in a concentration dependent manner so that at 100 μM at least 50% reduction of synthesis occurred (Table 2). Compound **2** reduced RNA synthesis 75% at 100 μM; yet **3** and **8** actually stimulated RNA synthesis ~50% at this concentration. Compound **8** at 25 μM caused an elevated level of protein synthesis and then caused a 23% reduction of protein synthesis at 100 μM for 60 min. Compounds **3** at 50 and 100 μM and **8** at all concentrations stimulated protein synthesis. DNA polymerase α activity was reduced 30% by compound **2** and 19% by compound **8**. m-RNA polymerase activity was suppressed marginally, 18% by compound **2** and 22% by compound **8**. r-RNA polymerase activity was reduced 50% by compound **2**, 46% by compound **3** and 38% by compound **8**. t-RNA polymerase activity was not significantly affected by the agents. Ribonucleoside reductase activity was only marginally reduced 6–16% by the agents at 100 μM. Dihydrofolate reductase activity was stimulated at lower concentrations of the agents; yet, only compound **8** caused a 20% reduction of activity at 100 μM after 60 min incubation.

Purine *de novo* synthesis was significantly inhibited by compounds **2**, **3** and **8** after 60 min. Both PRPP amido-transferase and IMP dehydrogenase activities were markedly reduced by all three compounds. *De novo* pyrimidine synthesis was not suppressed by the agents over 60 min and neither were the individual enzyme activities of the pathway, i.e. carbamyl phosphate synthetase, aspartate transcarbamylase and thymidylate synthetase. Thymidine kinase activity was reduced 19% by compound **8**. TMP kinase activity was suppressed 23% by compound **2** and 21% by compound **8** at 100 μM and TDP kinase activity was reduced 16–19% at 100 μM of the agents after 60 min. All three agents reduced d[ATP], d[GTP] and d[CTP] pool levels and d[TTP] pool levels were actually elevated by all three agents at 100 μM.

Studies with ct-DNA indicated that the agents did not act directly on the nucleoside bases of DNA based on negative finding after 24 hr. incubation at 100 μM, i.e. no changes in DNA viscosity, thermal denaturation or uv absorption from 220 to 340 nm. $Tmolt_3$ DNA strand scission studies demonstrated no significant DNA fragmentation after 24 hr. incubation of agents at 100 μM (data not shown).

Discussion

Several of the 4-hydroxyprolylglycinamide, N-cbz-4-substituted-prolylglycinamides and N-cbz-prolyl-4-substituted-prolylglycinamides at 8 mg/kg/day were active *in vivo* and were comparable to the effects of 6MP at 1 mg/kg/day and 5FU at 25 mg/kg/day in their ability to suppress Ehrlich ascites carcinoma tumor growth, >90% inhibition of tumor growth. Compound **1** with no cbz moiety was the most active in the *in vivo* screen. These data would suggest that *in vivo* efficacy of the agents as antineoplastic agents was evident and further investigation in *in vivo* tumor model is warranted in the future.

The compounds demonstrated potent inhibition of tumor cell growth in culture. There was no clear structure activity relationships for cytotoxic action. The unsubstituted 4-hydroxy derivative did demonstrate improved L1210 growth inhibition whereas in the $Tmolt_3$ screen the N-cbz-4-hydroxyprolylglycinamides, compounds **2**, **3**, **4**, demonstrated better activity. The substitution of the 2-carboxyethoxy, compounds **3** and **8** or the 4-carboxybutoxy, **4** and **9** at the 4-OH position of N-cbz-prolyl-4 or 4-substituted prolyl-glycermides afforded improved cytotoxicity in $Tmolt_3$ cells whereas the 2-carboxy-pyridylmethoxy substitutions, compounds **5** and **6** did not improve the activity. In the HeLa-S^3 screen the N-cbz-4-OH substituted prolylglycinamide, compound **2** afforded the best activity than any other substitution at the 4-position which led to less cytotoxic activity. Compound **1** with no cbz moiety was one of the more active compounds in the cytotoxic screens and positively correlated with the *in vivo* antineoplastic activity.

The major metabolic effect of the N-cbz-4-substituted prolylglycinamides in $Tmolt_3$ leukemia cells was the inhibition of DNA synthesis within 60 min. The inhibition of the activity of regulatory enzymes in the purine pathway correlated positively with the suppression of the purine biosynthesis and

DNA synthesis inhibition. The reduction in activities of both regulatory enzymes of this pathway, i.e. PRPP-amido transferase and IMP dehydrogenase, was probably additive and is very similar to the observed effects of 6-mercaptopurine. Some moderate reductions of nucleoside kinase, ribonucleoside reductase, DNA polymerase α and r-RNA polymerase activities afforded by the agents after 60 min probably was additive with the overall suppression of DNA synthesis but certainly these enzyme sites were not the major targets of the agents. These values were significantly different from the control values, but the reduction was not of a magnitude to account for the reduction in DNA synthesis. The DNA molecule itself did not appear to be the target of the agents since no intercalation, binding to nucleoside bases, cross linking or DNA fragmentation was evident after 60 min incubation. Thus, it can be concluded that the N-cbz-4-substituted prolylglycinamides are antimetabolite antineoplastic agents. The d[NTP] pool levels of $Tmolt_3$ leukemia cells were altered by drug treatment for 60 min. Reduction by the derivatives of *de novo* purine synthesis would ultimately lower d[ATP] and d[GTP] pools in the cell. It is remarkable that this reduction was evident after only 60 min. The fact that d[TTP] pools were not reduced is consistent with the fact that the agents did not suppress *de novo* pyrimidine synthesis, including the activities of the early regulatory enzymes of the pathway, i.e. carbamyl phosphate synthetase and aspartate transcarbamylase activities. Actually thymidylate synthetase activity was slightly elevated and probably accounts for the elevated dTTP levels observed at the expense of dCTP pool levels. Nucleotide levels may also be lowered due to the agents slightly reducing ribonucleoside reductase and nucleoside kinase activities although the effects were marginal. These d[NTP] pool levels are the net effects of the agents on their *de novo* synthesis, incorporation of bases into a new strand of DNA and the nucleotide retrieval pathways. The fact that DNA polymerase α activity was slightly suppressed would cause slightly elevated levels of d[NTP] pools whereas inhibition of the purine pathway would ultimately lead to reduction of d[NTP] synthesis. Reductions of d[NTP] pools are usually observed early because of their low level in mammalian cells compared to r[NTP] pools.

The boron containing di- and tri-peptides of glycine, alanine, betaine, phenylalanine, and tyrosine (Hall et al., 1993), as well as the carbobenzoxy(cbz)phenylalanine vinyl esters and 1,2-dibromoethyl esters demonstrated similar cytotoxicity/antineoplastic (Loeffler et al., 1977) activities as did the di- and tripeptide 4-substituted hydroxyproline derivatives. The latter compounds appeared more potent in the *in vivo* screen with more similar activity to the clinical antimetabolites, 6-MP and 5-FU. Mode of action studies have not been completed on many of the antimetabolite peptides excluding those which are alkylating agents. N-acetyl-p-boron-phenyl-alanyl-phenylamine-methyl ester inhibited L-1210 DNA, RNA and protein syntheses (Hall et al., 1993). It was a potent inhibitor of L-1210 IMP dehydrogenase and ribonucleoside reductase activities but not PRPP-amido transferase activity. The N-benzyloxycarbonyl-L-phenylalanine esters inhibited Ehrlich ascites carcinoma DNA and protein syntheses after *in vivo* treatment for 3

days at 8 mg/kg/day (Hall et al., 1978). Thymidylate synthetase and nucleoside kinase activities were reduced by these derivatives. Thus, it appears that substituted di- and tri-peptides can act as antimetabolites and the di- and tripeptide 4-substituted hydroxyproline derivatives may be exclusively directed towards inhibition of purine *de novo* synthesis.

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