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Antitumor action of *N*-(2-chloroethyl)-*N*-nitrosocarbamoyl derivatives of biologically active polypeptide hormone fragments

A. Jeney[1], L. Kopper[1], P. Nagy[1], K. Lapis[1], Helga Süli-Vargha[2], and K. Medzihradszky[2]

[1] 1st. Institute of Pathology and Experimental Cancer Research, Semmelweis Medical University, Budapest, Hungary
[2] Institute of Organic Chemistry, Eötvös Loránd University Budapest, Hungary

Summary. The antitumor action of the 2-chloroethylnitrosocarbamoyl derivatives of peptides related to the 9–13 amino acid residues of α-MSH/ACTH and of the C-terminal tetrapeptide analogue of gastrin have been investigated. Series of 2-chloroethylnitrosoureas attached to amino acids, di-, tri-, tetra-, or pentapeptides were examined in a primary screening system. Among these compounds the Pro-Val-, Lys-Pro-Val-, and Trp-Gly-Lys-Pro-Val-containing 2-chloroethylnitrosocarbamoyl groups were the most effective in the L_{1210} system. The human melanoma xenograft line was also affected by these agents, while colorectal xenografts were insensitive. A combination of tripeptide-2-chloroethyl-nitrosourea with BCNU induced more than additive growth inhibition of L_{1210} leukemia.

Introduction

Amino acids have been linked to the cytotoxic chloroethylamino or chloroethylnitrosocarbamoyl groups in efforts to obtain antitumor drugs with less toxic side effects [1, 2, 5, 11, 9]. The use of peptides as carriers of cytotoxic groups has also been investigated [2, 3, 6, 9, 10]. Since peptides have been recognized as important regulatory elements of cell growth and certain organ specific functions, it has been assumed that the synthesis of *N*-(2-chloroethyl)-*N*-nitrosocarbamoyl derivatives of biologically active polypeptide hormone fragments may result in antitumor drugs with certain advantageous properties [8]. In this communication the antitumor actions of the 2-chloroethylnitrosocarbamoyl derivatives of certain amino acids and peptides related to the primary structure of α-MSH/ACTH and gastrin are reported.

Materials and methods

Substances. All compounds tested were synthesized and analyzed as previously reported [8]. The compounds were homogeneous by both thin-layer chromatography and elemental analysis and showed absorbance characteristics for the *N*-nitroso bond (λ max.: 400 nm). Their decomposition rates were determined by measuring the decrease of extinction at λ max. in 0.1 *M* Na phosphate buffer (pH 7.4) containing 5% ethanol at 37 °C.

Offprint requests to: A. Jeney

Animals and tumors. C57BL × DBA_2F_1 hybrid mice of either sex bred in our institute were inoculated with 10^6 cells of L_{1210} leukemia IP at 6–10 weeks of age. The L_{1210} was maintained by weekly transplantation in DBA_2 mice. Compounds in the amount equivalent to the dose on a per kilogram of body weight basis were dissolved in saline except for compounds nos. 1, 2, 4, 8, and 10 (Table 1), which were dissolved in 0.2 ml dimethylsulfoxide and diluted to 10 ml with 0.9% NaCl. Treatment consisted of single IP injections at the times indicated in a volume of 0.1 ml per 10 g body weight. The percentage increase in life-span (ILS %) of treated animals was compared with the life-span in untreated tumor bearers and was calculated as follows: $\text{ILS\%} = \frac{T-C}{C} \times 100$; (T and C = median survival in days for treated and control groups, respectively).

B_{16} melanoma and S_{180} fragments were transplanted SC into the flank of C57BL and Swiss mice, respectively. Lewis lung tumor was inoculated IM into C57BL mice. Human melanoma and colorectal tumor xenograft lines growing SC were established and maintained in our institute in artificially immunosuppressed inbred CBA mice. Drug treatment and quantitative evaluation of tumor growth were performed as described in Table 2 [4].

Results and discussion

L_{1210} leukemia-bearing mice were treated with various *N*-chloroethyl/*N*-nitrosocarbamoyl (R) derivatives of peptides related to the 9–13 amino acid residues of α-MSH/ACTH and to the C-terminal tetrapeptide analogue of gastrin. The life-span of treated mice is shown in Table 1. The chemical decomposition rates of these compounds were similar (15–20 min), except for those with a proline terminus (nos. 2 and 4), where the 2-chloroethylnitrosocarbamoyl group is linked to a tertiary nitrogen atom. It appears that chemical reactivity determines the toxicity of those compounds as well, because R-Pro-NH_2 and R-Pro-Val-NH_2 (nos. and 4) exerted lethality at 0.806 and 0.560 mmol/kg respectively, whereas R-Val-NH_2 and R-Lys-NH_2 killed the mice at much lower (0.1–0.16 mmol/kg) dose levels.

It is possible that the peptide moiety may also modify toxic behavior of the nitrosourea compounds. This notion seems to be supported by studying the biological activity of R-Trp-Gly-Lys-Pro-Val-NH_2 containing the 9–13 ami-

Table 1. Antitumor actions of α-MSH/ACTH-related peptide derivatives of chloroethylnitrosoureas on L_{1210}-leukemia in vivo

No. of compounds:	Doses[a]		ILS (%)	Chemical half-life (min)
	(mg/kg)	(mmol/kg)		
1. *R*-Val-NH_2[b]	10	0.04	141	15.5
	20	0.08	212	
	40	0.16	Toxic	
2. *R*-Pro-NH_2	50	0.201	39	255.5
	100	0.403	420	
	200	0.806	Toxic	
3. *R*-Lys-NH_2HCl	10	0.031	20	19.7
	20	0.063	28	
	34	0.107	Toxic	
4. *R*-Pro-Val-NH_2	20	0.056	30	187.2
	40	0.112	75	
	80	0.230	136	
	100	0.280	80% survival[c]	
	200	0.560	Toxic	
5. *R*-Lys-Pro-Val-NH_2HCl	12.5	0.024	50	29.8
	25.0	0.048	75	
	50	0.097	100	
	100	0.195	263	
	150	0.254	60% survival	
	200	0.390	Toxic	
6. *R*-Gly-Lys-Pro-Val-NH_2HCl	25	0.043	27	25.8
	50	0.087	48	
	100	0.175	100	
	200	0.351	Toxic	
7. *R*-Trp-Gly-Lys-Pro-Val-NH_2HCl	12	0.015	20	19.9
	25	0.030	100	
	50	0.061	300	
	100	0.122	60%[a]	
	200	0.244	60%[a]	
	300	0.366	160	
8. *R*-Trp-Leu-Asp-Phe-NH_2	50	0.070	0	n.d.
	100	0.140	40	
	150	0.210	230	
	200	0.280	Toxic	
9. *R*-Tyr-OCH_3	175	0.053	0	n.d.
	35	0.106	50	
	70	0.212	Toxic	
10. α, ε-Bis-(2-chloroethyl)-carbamoyl-Lys-Pro-Val-NH_2	10	0.018	0	n.d.
	20	0.036	0	
	80	0.144	0	
11. H-Lys-Pro-Val-NH_2.$2CH_2COOH$	50	0.109	0	n.d.
12. H-Lys-Pro-Gly-Lys-Pro-Val-NH_2	50	0.085	0	n.d.

[a] Treatment was administered IP on the first day after transplantation

[b] $R = Cl\text{-}CH_2CH_2\text{-}N(NO)\text{-}C(=O)\text{-}$

[c] Survival = ILS % > 600

n.d. = not determined

Table 2. Growth-inhibitory action of 2-chloroethylnitrosocarbamoyl derivatives of some α-MSH/ACTH-related peptides on human tumor xenografts

Tumors	Compounds: $R = ClCH_2CH_2-N(-NO)-C-$	Tumor growth inhibition[a]				
		10 mg/kg	20 mg/kg	50 mg/kg	100 mg/kg	5 × 20 mg/kg
HT18 Melanoma	BCNU	59%	83%	–	–	–
	R–Pro–Val–NH_2	–	–	–	84%	62%
	R–Lys–Pro–Val–NH_2HCl	–	–	40%	77%	68%
	R–Gly–Lys–Pro–Val–NH_2HCl	–	–	57%	52%	–
	R–Trp–Gly–Lys–Pro–Val–NH_2HCl	–	–	65%	87%	–
HT17 Colon ca.	*R*–Lys–Pro–Val–NH_2HCl	–	–	–	13%	5%
HT22 Colon ca.	*R*–Lys–Pro–Val–NH_2HCl	–	–	–	52%	9%
HT59 Colon ca.	*R*–Pro–Val–NH_2	–	–	–	42%	–
HT22 Pancreas ca.	*R*–Trp–Gly–Lys–Pro–Val–NH_2HCl	–	–	–	38%	–

[a] $TGI = \left(1 - \frac{TV}{CV}\right) \times 100$ TV, volume of treated tumor; CV, volume of control tumors

[b] Tumors were treated IP at about 8–10 mm in diameter; the repeated injections were given on 5 consecutive days

no acid sequence in α-MSH or in ACTH. This compound showed no lethality at the highest dose given (0.366 mmol/kg), but was able to induce a remarkable cure rate at the dose of 0.122 mmol/kg. Similarly, administration of R-Pro-Val-NH_2 and R-Lys-Pro-Val-NH_2 (nos 4 and 5) also resulted in substantial prolongation of life-span and 80% cure rates. However, the amino acid and the tetrapeptide derivatives exerted a much lower degree of antitumor action. Some of them (nos. 1, 2, 3, 6) contained the amino acids of the 9–13 peptide fragment in MSH/ACTH, but others (nos. 8 and 9) were unrelated. The bifunctional 2-chloroethylcarbamoyl derivative of Lys-Pro-Val (10) and the peptide carrier alone (nos. 11 and 12) did not cause any prolongation of life-span in L1210-bearing mice.

Among the series of 2-chloroethyl-nitrosourea compounds, R-Lys-Pro-Val-NH_2 (5) was subjected to further examination in Lewis lung tumor, sarcoma 180, B_{16} melanoma, and three human tumor xenografts. In sarcoma 180 there was no response at all, and Lewis lung tumor showed only modest susceptibility (less than 25% increase in survival time) to this compound (data not shown). In contrast, the growth of B_{16} melanoma was retarded for a long period (Fig. 1). An amelanotic human melanoma xenograft (HT 18) was also very sensitive to R-Lys-Pro-Val-NH_2. The doubling time of this tumor was more than twice as long in the treated as in the control tumor. The ability of three other *N*(2-chloroethyl)-*N*-nitrosocarbamoyl peptide to reduce the growth of the HT18 melanoma xenograft was compared with that of the R-Lys-Pro-Val-NH_2 derivative. As shown in Table 2, the R-Gly-Lys-Pro-Val-NH_2 derivative was the least effective of the compounds tested. Beside the HT18 melanoma the growth of certain other human tumor xenografts was also decreased, but to a lesser extent (Table 2).

With reference to the molecular weights of the compounds, R-Trp-Gly-Lys-Pro-Val-NH_2 appeared to the most potent antitumor drug. The same level of growth in-

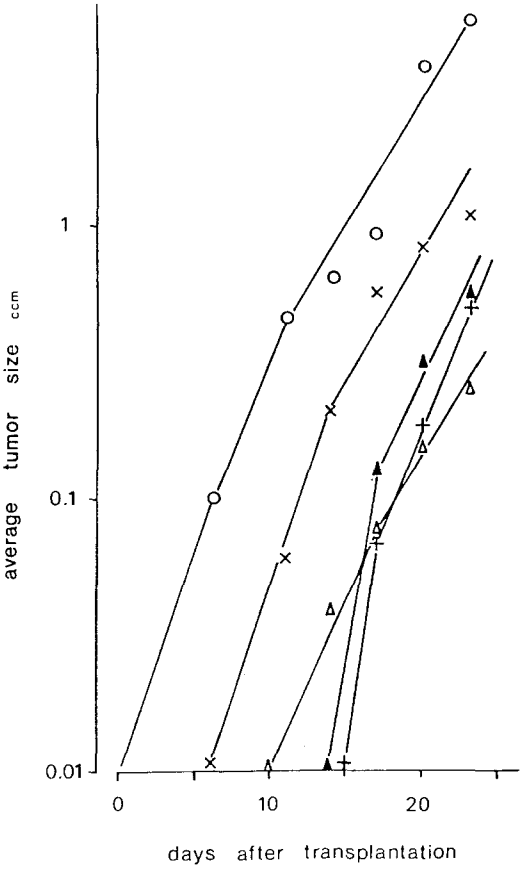

Fig. 1. Tumor growth-inhibitory action of *N*-(2-chloroethyl)-*N*-nitrosocarbamoyl-Lys-Pro-Val-NH_2 (no. 5) and 1,3- bis (2-chloroethyl)-1-nitrosourea (BCNU) on B16 melanoma. 0———0, controls› ▲———▲, compound no. 5, 100 mg/kg; △———△, compound no. 5 5 × 20 mg/kg; +———+, BCNU 20 mg/kg; X–X BCNU 5 × 4 mg/kg

Table 3. Combination therapy with *N*-(2-chloroethyl)*N*-nitrosocarbamoyl-Lys-Pro-Val-NH_2 (no. 5) on L1210 mice

Compound	Doses	Treatment time after transplantation (days)	Increased life-span %	
			Calculated[a]	Observed
No. 5 + BCNU	50 mg/kg + 5 mg/kg	1	87 + 25	325
	25 mg/kg + 10 mg/kg	2	44 + 77	70% cured[b]
	12.5 mg/kg + 20 mg/kg	1	50 + 110	325
	50 mg/kg + 20 mg/kg	3	33 + 70	60% cured[b]
No. 5 + cyclophosphamide				
	25 mg/kg + 50 mg/kg	2	44 + 44	77

[a] After single-agent therapy
[b] Survival = ILS% > 600

hibitory action was achieved with approximately 43% of the molecules required in the case of R-Pro-Val-NH_2. This implies that a substantially smaller number of chloroethyl-nitrosocarbamoyl groups can cause inhibition of cell proliferation, probably because the Trp-Gly-Lys-Pro-Val moiety either facilitates the selective drug uptake into the melanoma or carries the cytotoxic chloroethylnitroso group more readily to the intracellular target sites.

When a new member of an established class of antitumor drugs has been developed, the question remains as to whether it represents a real advantage over the parent compounds. Conclusions from comparative studies of antitumor efficacy may be misleading. One appropriate approach to this problem is to apply the test compounds in combination with other antitumor drugs. It may be presumed that compounds with different molecular mechanisms offer more than additive antitumor action in combination therapy, whereas the joint action of drugs acting on the same target is only additive. Therefore R-Lys-Pro-Val-NH_2 (5) and BCNU were administered in various dose ratios and the observed antitumor efficacies were compared with the calculated one (i.e., the sum of the antitumor action of the drugs after single-agent therapy). Table 3 shows that R-Lys-Pro-Val-NH_2 and BCNU in combination resulted in a more than additive antitumor action.

.The illustrated synergism may be an indication that the mechanism of the 2-chloroethyl-nitrosocarbamoyl derivatives of the α-MSH/ACTH-related peptides are different in certain aspects from BCNU. Our current efforts are directed toward evaluating this possibility and to elucidating the role of the peptide carrier in the antitumor action of these novel 2-chloroethyl-nitrosourea compounds.

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