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4-Aroyl-1-nitrosohydrazinecarboxamides: synthesis, *in vivo* and *in vitro* antitumor activity

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Summary — 4-Benzoyl-1-(2-chloroethyl)-1-nitrosohydrazinecarboxamide (**I**), 1-(2-chloroethyl)-1-nitroso-4-phenylacetylhydrazinecarboxamide (**II**), 1-(2-chloroethyl)-4-(2-hydroxybenzoyl)-1-nitrosohydrazinecarboxamide (**III**), 4-(4-aminobenzoyl)-1-(2-chloroethyl)-1-nitrosohydrazinecarboxamide (**IV**), 4-(4-chlorobenzoyl)-1-(2-chloroethyl)-1-nitrosohydrazinecarboxamide (**V**), all novel class nitrosoureas, were synthesized. All five compounds showed a dose-dependent activity against leukemias L1210, P388 and Ehrlich ascites tumor (EAT). Compound **I** was superior, yielding T/C% values of 400%, resulting in many cures, reaching 100% in EAT mice. The effect of the above substances on the incorporation of radioactive precursors into DNA, RNA and proteins of EAT cells was investigated. It was found that treatment of cells with 50 μg/ml of the compounds causes significant inhibition (approximately 70%) of the incorporation of ^{3}H-thymidine into DNA. Finally, the effect of these compounds on sister chromatid exchange (SCE) values and on cell kinetics in cultured human lymphocytes was studied. Compound **I** was found to be the most effective in causing markedly increased SCE values and cell division delays.

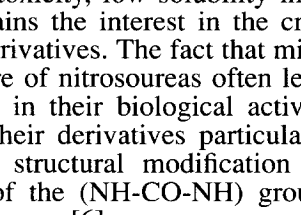
4-aroyl-1-nitrosohydrazinecarboxamide / nitrosoureas / antitumor activity / leukemia L1210 / leukemia P388 / Ehrlich ascites tumor / sister chromatid exchange assay

Introduction

Since the emergence of 1,3-bis-(2-chloroethyl)-1-nitrosourea (BCNU) and 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU) as useful antitumor agents in treating a variety of human malignancies and solid tumors, a large number of nitrosourea derivatives that are highly active in experimental tumor systems have been reported [1–3].

The nitrosoureas have a number of shortcomings such as high toxicity, low solubility in water, etc [4, 5]. This explains the interest in the creation of new nitrosourea derivatives. The fact that minimal changes in the structure of nitrosoureas often lead to considerable changes in their biological activity makes the synthesis of their derivatives particularly promising. One possible structural modification could be the replacement of the (NH-CO-NH) group by a (NH-CO-NH-NH) group [6].

Bearing in mind the high biological activity (including antitumor) of the aroylhydrazides, we aimed at obtaining a new group of 4-aroyl-1-nitrosohydrazinecarboxamides and examined them for antitumor effects. The structure of the newly obtained compounds is shown in scheme 1. They exhibited relatively low toxicity compared with that of CCNU and high antitumor effect against L1210, P388 and



Where: Ar = C_6H_5 **I**. $C_6H_5CH_2$ **II**. 2-HOC_6H_4 **III**. 4-$H_2NC_6H_4$ **IV**. 4-ClC_6H_4 **V**.

Scheme 1. Structure of the newly obtained compounds.

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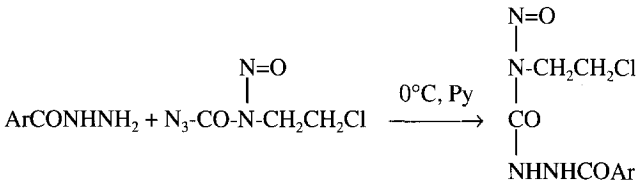
EAT. They inhibited DNA, RNA and protein synthesis and caused increased SCE values and cell division delays.

Chemistry

Compounds (I–V) were synthesized by direct reaction between aroylhydrazides and *N*-chloroethyl-*N*-nitrosocarbamoylazide (scheme 2) as described in [7].

The reaction was carried out in anhydrous pyridine, at 0°C with constant stirring for several hours. The end of the reaction was controlled by TLC (silica gel, mobile phase chloroform:methanol = 9:1 and 95:5). The compounds obtained were pale yellow crystals with high mp, easily soluble in methanol, ethanol, benzene and poorly soluble in *n*-hexane. Compounds **I, II** and **V** expressed relatively low solubility (isolated according to *Method A*, described in *Experimental protocols*), while **III** and **IV** were readily soluble in water (*Methods B* and *C*). This should be borne in mind for their removal from the reaction mixture. The *N*-nitrosohydrazinecarboxamides were obtained in high yield (75–82%). The structure was confirmed by elemental analysis, IR, NMR and mass spectroscopy (details about NMR and mass-spectra will be published in a separate paper – G Muller *et al*). It should be pointed out that these compounds were rich in nitrogen and can be explosively decomposed by combustion which could lead to improper values of elemental analysis. Their alkylating properties were evaluated by using NBP-tests [8].



Scheme 2. Synthesis of 4-aroyl-1-nitrosohydrazinecarboxamides **I–V**.

Results and discussion

In vivo experiments

The toxicity of compounds **I–V** was assessed from the lethality in BALB/c mice within 30 days and the LD_{50} values were estimated graphically. LD_{50} doses were 70, 68, 66, 66 and 68 mg/kg bw for **I–V** respectively.

The results of the *in vivo* antitumor activity on leukemias P388, L1210 and EAT of all synthesized compounds are reported in table I. For all compounds the following doses were used: 18, 9, 4.5 mg/kg bw

Table I. Antitumor activity of compounds **I–V** on leukemias P388, L1210 and EAT.

Compound	*Treatment schedule*	*P388*			*L1210*			*EAT*		
		MST (days)	*T/C%*	*Cures on d 60*	*MST (days)*	*T/C%*	*Cures on d 60*	*MST (days)*	*T/C%*	*Cures on d 60*
Control	–	12	100	0	8.75	100	0	21	100	0
I	D	22	183	1/6	13	148	0/6	–	–	6/6
	D/2 x 3	48	400	3/6	23	264	2/6	–	–	6/6
	D/4 x 7	22.5	187	0/6	17	194	1/6	43	205	3/6
II	D	20.5	170	0/6	11	126	0/6	58	276	3/6
	D/2 x 3	35	292	2/6	18.5	211	1/6	73.5	350	5/6
	D/4 x 7	26.5	221	0/6	14	160	1/6	50	238	2/6
III	D	15	125	0/6	10.5	120	0/6	38	181	0/6
	D/2 x 3	17	142	0/6	13	148	0/6	45	214	1/6
	D/4 x 7	16.5	137	0/6	12.5	143	0/6	32	152	0/6
IV	D	18	150	1/6	11	126	0/6	64	305	2/6
	D/2 x 3	30	250	2/6	15	171	1/6	77	367	5/6
	D/4 x 7	26	217	0/6	13.5	154	0/6	47	224	3/6
V	D	13	108	0/6	9.5	108	0/6	28.5	136	0/6
	D/2 x 3	15	125	0/6	10	114	0/6	33	157	0/6
	D/4 x 7	12	100	0/6	9	103	0/6	25	119	0/6

MST = mean survival time; D, 18 mg/kg; D/2 x 3, 9 mg/kg; D/4 x 7, 4.5 mg/kg.

based on single (D), intermittent (D/2 x 3) and consecutive (D/4 x 7) dose schedules. We were able to discern 3 out of 5 compounds that displayed activity against leukemias P388, L1210 and EAT. These substances were found to considerably increase the lifespan of the tumor bearing animals and even produce 100% cures.

Compound I
This compound was more or less effective against all 3 of the transplantable tumors. It was very effective against EAT, producing 6/6 cures, when the treatment schedule was on day 1 and days 1, 4, 7 (D, D/2 x 3). P388 responded better than L1210, producing 3/6 and 2/6 cures and increasing the lifespan by 400 and 264%, respectively (non-cured mice) when the intermittent dose was used.

Compound II
Administration of this compound on intermittent schedule caused 2/6 and 1/6 cures, T/C = 292 and 211% on P388 and L1210, respectively. However, EAT gave much better results, providing 5/6 cures and increasing the lifespan of non-cured animals by 350%.

Compound IV
Using D/2 x 3 treatment schedule this compound achieved 5/6 cures on EAT, 2/6 and 1/6 cures on P388 and L1210, respectively. The extension of lifespan in non-cured animals was also considerable.

Compounds III and V
The effect obtained with these compounds was similar for all treatment schedules. The tests against P388 and L1210 leukemias produced no cures and no significant increase in lifespan. However, **III** resulted in 1/6 cures and T/C = 214% on EAT, when it was administered on days 1, 4, 7.

Three out of five evaluated compounds displayed strong antitumor activity *in vivo*. It can be seen from the results that compound **I** is the most active, causing 100, 50 and 33% cures on EAT, P388 and L1210, respectively. In these experiments the intermittent (D/2 x 3) treatment schedule gave better results than the single dose (D) and was much better than the consecutive dose (D/4 x 7). Compound **III** displayed activity only on EAT, achieving 17% cures and 214% increase in life span of non-cured animals. Compound **V** was not effective against all three of the tumors used in this study.

In vitro experiments

The effects of compounds **I–V** on DNA, RNA and protein synthesis are shown in table II. EAT cells incubated for 1 h in 199 medium supplemented with calf serum were maintained in good condition as judged by the Trypan blue viability test. The presence of 5 μg/ml of 4-aroyl-1-nitrosohydrazinecarboxamides affected the cells non significantly, since less than 2% of dead cells were counted after 1 h incubation. The above compounds mainly inhibited the incorporation of labelled thymidine into DNA. There was little difference in inhibition of DNA synthesis, as determined by ^{3}H-thymidine incorporation into DNA, following administration of the compounds.

Table II. The effect of compounds **I–V** on DNA, RNA and protein synthesis of EAT cells.

Compd	*^{3}H-Thymydine (%)*		*^{3}H-Uridine (%)*		*^{3}H-Leucine (%)*	
	cpm	*Inhib*	*cpm*	*Inhib*	*cpm*	*Inhib*
Control	2040	0	3754	0	749	0
I	703	66	3832	25	482	36
II	620	70	3470	18	417	45
III	620	70	2523	33	544	28
Control	5304	0	4370	0	1006	0
IV	1722	68	2669	49	792	21
V	3089	42	3054	31	824	18

The comparison of the activities of the compounds led to the following conclusions: the above compounds mainly inhibited the incorporation of labelled thymidine into DNA. Compound **I** exhibited a 66% inhibitory effect on DNA synthesis, whereas the effect on RNA and protein synthesis was much smaller. Compounds **II** and **III** demonstrated a slightly higher effect, causing 70% inhibition of DNA synthesis. Compound **IV** caused 68% inhibition of DNA synthesis. The smaller effect on DNA synthesis was caused by compound **V**, producing 42% inhibition.

SCE formation

The results of SCE assay are presented in table III. Compounds **I** and **IV** increase SCE rates at all concentrations tested and appear as the most effective. The increases were directly related to the concentrations used. Next in order of effectiveness was **II** with **III** following closely. Compound **V** doubled the control rate of SCEs only at the higher concentration tested and appeared to be less effective. All the chemicals appeared to be inducers of cell division delays. There are findings indicating that the effectiveness in SCE induction by antitumor alkylating agents in cancer rodent cells *in vitro* [9] and *in vivo* [10] can be positively correlated with the *in vivo* tumor response to these agents and suggest that SCE assay could be used to predict both the

Table III. Induction of SCEs and cell division delays in human lymphocytes.

Compd	*Concentration (μM)*	*SCEs ± SE* [a]	*Range*	*PRI* [b]
Control	–	8.26 ± 0.87	(5–13)	2.09
I	10	16.14 ± 1.45	(4–29)	1.82
	20	26.76 ± 1.64	(8–35)	1.90
	30	38.98 ± 1.71	(15–70)	1.86
II	10	12.24 ± 1.27	(3–23)	1.81
	20	22.83 ± 1.06	(17–33)	1.80
	30	23.98 ± 1.52	(17–32)	1.75
III	10	13.00 ± 1.16	(6–20)	1.91
	20	16.03 ± 1.38	(9–25)	1.86
	30	24.16 ± 1.80	(14–34)	1.77
IV	10	19.85 ± 2.01	(9–30)	1.27
	20	31.16 ± 2.12	(14–48)	1.23
	30	38.15 ± 2.86	(27–59)	1.17
V	10	14.30 ± 1.37	(7–21)	1.78
	20	13.77 ± 1.54	(7–27)	1.80
	30	19.80 ± 1.60	(8–28)	1.47

[a]For mean SCE values 50 cells were scored for each culture; [b]proliferation rate indices (PRI) were calculated as (M_1 + $2M_2$ + $3M_3$ +)/100, where M_1 is the percent value of cells in the 1st, M_2 in the 2nd and M_3 + in the 3rd and higher divisions; for PRIs 100 cells were scored.

sensitivity of human tumor cells to chemotherapeutic agents and the heterogeneity of drug sensitivity within individual tumors [11].

In the present experiments **V** is comparatively the less effective inducer of SCEs and of cell division delays which correlates well with its limited antitumor activity. Compounds **I–IV** appear as effective inducers of SCEs and of cell division delays demonstrating antitumor activity as well. Studies are underway to clarify whether an improvement of the structure, in order to achieve better activity, is possible.

Finally, the results of the *in vivo* antitumor activity demonstrated that **I** was the most effective, followed by **II** and **IV**. The results of SCE assay demonstrated that **I** and **IV** were the most effective. It could be mentioned that **I** was also the most effective when it was tested in *Salmonella typhimurium* assay [12]. In contrast, no differences were observed in the activity of **I–IV**, when inhibition of DNA synthesis *in vitro* was studied, whereas **V** had a much smaller effect. Apparently, in this case, the structure of the residue (R) played no important role on the expression of the inhibitory effect.

Experimental protocols

*General procedure for synthesis of compounds **I–V***

A solution of 2-chloroethylnitrosocarbamoylazide (1.06 g, 6.1 mmol) [7] in dry ether (10 ml) was added dropwise to a continuously stirred solution, cooled to 0°C, of the corresponding aroylhydrazide (5 mmol) in anhydrous pyridine (10 ml). When no more aroylhydrazide could be detected by TLC (precoated silica gel glass plates, layer thickness 0.25 mm, F_{254}, Merck, Germany), the reaction mixture was poured into cold water (30 ml). The isolation of the corresponding substances was performed according to their solubility in water.

Melting points were determined on a Büchi 535 (Switzerland) capillary melting point apparatus. IR spectra were recorded as specified with a Beckman IR-33 (USA) spectrophotometer. NMR spectra (300 MHz) were recorded in acetone-d_6 with a Bruker AMX-300 (Germany) spectrometer. Chemical shifts were reported in parts per million (ppm) (δ). Mass spectra were recorded with a Jeol JMS D 100 spectrometer. The IR, NMR and mass spectra of all new compounds were consistent with the expected structures.

*4-Benzoyl-1-(2-chloroethyl)-1-nitrosohydrazinecarboxamide **I***
After pouring the compound into the ice-cold water the precipitate formed was filtered, washed with cold water and recrystallized from methanol-water *(Method A)*. Compound **I** was obtained as a pale yellow powder, mp 168°C; R_f=0.72 (chloroform:methanol = 9:1); yield 82%; IR (Nujol, cm^{-1}) 3200 (NH), 1725 (C=O), 1529 (CNH), 1480 (N=O); ^{1}H-NMR: 9.93 (s, 2H, NHNH), 7.98–7.33 (m, Ar H), 4.20 (t, J = 6.4 Hz, 2H, $N(NO)CH_2$), 3.63 (t, J = 6.4 Hz, 2H, CH_2Cl); MS (low resolution) *m/e* 271 (M^+ +H), 166 (M^+-C_6H_5CO), 163 (M^+-$N(NO)CH_2CH_2Cl$); M_r 270.20 calculated for $C_{10}H_{11}N_4O_3Cl$ (C, H, N).

*1-(2-Chloroethyl)-1-nitroso-4-phenylacetylhydrazinecarboxamide **II***
This was obtained (see *Method A*) as pale yellow crystals, mp 130°C; R_f=0.70 (chloroform:methanol = 9:1); yield 80%; IR (Nujol, cm^{-1}) 3237 (NH), 1754 (C=O), 1531 (CNH), 1488 (N=O); ^{1}H-NMR: 9.76–9.48 (d, 2H, NHNH), 7.37–7.20 (m, Ar H), 4.16 (t, J = 6.2 Hz, 2H, $N(NO)CH_2$), 3.62 (d, J = 2.2 Hz, 2H, $ArCH_2$), 3.59 (d, J = 6.5 Hz, 2H, CH_2Cl); MS (low resolution) *m/e* 285 (M^+ +H), 177 (M^+-$N(NO)CH_2CH_2Cl$), 166 (M^+- $C_6H_5CH_2CO$); M_r 284.71 calculated for $C_{11}H_{13}N_4O_3Cl$ (C, H, N).

*1-(2-Chloroethyl)-4-(2-hydroxybenzoyl)-1-nitrosohydrazinecarboxamide **III***
After pouring into the ice-cold water, the solution formed was extracted with ether. The ether extract was washed consecutively with ice-cold 2 N HCl (2 x 30 ml), 5% solution of $NaHCO_3$ (2 x 30 ml), saturated solution of NaCl, dried over $MgSO_4$ and evaporated *in vacuo*. The dry residue was recrystallized from methanol-*n*-hexane *(Method B)*. The compound was obtained as pale yellow crystals, mp 118°C; R_f = 0.67 (chloroform:methanol = 9:1); yield 75%; IR (Nujol, cm^{-1}) 3400–3273 (OH, NH), 1737 (C=O), 1537 (CNH), 1488 (N=O); ^{1}H-NMR: 11.80 (s, 1H, ArOH), 10.31–10.05 (d, 2H, NHNH), 7.96–6.91 (m, Ar H), 4.20 (t, J = 6.3 Hz, 2H, $N(NO)CH_2$), 3.64 (t, J = 6.3 Hz, 2H, CH_2Cl); MS (low resolution) *m/e* 287 (M^+ +H), 179 (M^+ - $N(NO)CH_2CH_2Cl$), 166 (M^+-OHC_6H_4CO); M_r 286.69 calculated for $C_{10}H_{11}N_4O_4Cl$ (C, H, N).

4-(2-Aminobenzoyl)-1-(2-chloroethyl)-1-nitrosohydrazinecarboxamide ***IV***
After pouring into the ice-cold water the solution formed was extracted with ether. The ether extract was evaporated *in vacuo* to dryness (up to 35°C). The yellow oil obtained was treated twice in toluene and dried. The residue was recrystallized from methanol-*n*-hexane (method C) as yellow crystals, mp 170°C; R_f = 0.36 (chloroform:methanol = 95:5); yield 78%; IR (Nujol, cm^{-1}) 3470 (NH_2), 1735 (C=O), 1533 (CNH), 1480 (N=O); NMR: 9.73–9.52 (d, 2H, NHNH), 7.94–6.67 (m, Ar H), 5.29 (s, 2H, $ArNH_2$), 4.19 (t, J = 6.5 Hz, 2H, $N(NO)CH_2$), 3.62 (t, J = 6.4 Hz, 2H, CH_2Cl); MS (low resolution) *m/e* 286 (M^+ +H), 178 (M^+-$N(NO)CH_2CH_2Cl$), 166 (M^+-$NH_2C_6H_4CO$); M_r 285.70 calculated for $C_{10}H_{12}N_5O_3Cl$ (C, H, N).

4-(4-Chlorobenzoyl)-1-(2-chloroethyl)-1-nitrosohydrazinecarboxamide ***V***
This was obtained (see *Method A*) as pale yellow crystals, mp 147°C; R_f = 0.56 (chloroform:methanol = 95:5); yield 81%; IR (KBr, cm^{-1}) 3220 (NH), 1690 (C=O), 1529 (CNH), 1480 (N=O); ^{1}H-NMR: 10.05–9.96 (d, 2H, NHNH), 8.00–7.53 (m, Ar H), 4.20 (t, J = 6.6 Hz, 2H, $N(NO)CH_2$), 3.62 (t, J = 6.3 Hz, 2H, CH_2Cl); MS (low resolution) *m/e* 306 (M^+ +H), 197 (M^+-$N(NO)CH_2CH_2Cl$), 166 (M^+-ClC_6H_4CO); M_r 305.06 calculated for $C_{10}H_{10}N_4O_3Cl_2$ (C, H, N).

Biological methods

In vivo experiments

Mice tumors
Ehrlich ascites tumors, female BALB/c mice, weighing 20–25 g were used for the experiments and for carrying the tumor. For L1210 and P388 leukemias, DBAxC57BL (BDF_1) weighing 20–25 g were used.

Lymphoid L1210 and lymphocytic P388 leukemias were maintained in ascitic form in DBA/2 mice, by injection of 10^5 and 10^6 cells, respectively, at 7-day intervals, into the peritoneal cavity. Ehrlich ascites tumor (EAT) was grown in A mice by intraperitoneal injection of 7 x 10^6 cells [13].

Drugs
For intraperitoneal treatment, stock solutions of the compounds used in this study were made immediately before use. They were suspended in corn oil in the desired concentration following initial dissolution in a small amount of 10% dimethylsulfoxide. This concentration by itself produced no observable toxic effect.

Antitumor evaluation
EAT experiments started by implanting BALB/c mice with 2 x 10^6 cells/mouse.

Antileukemia experiments were initiated by implanting BDF_1 mice with the appropriate number of cells.

For all experiments the antitumor activity was assessed according to the following criteria: the percentage increase in median lifespan over the control (T/C%); and the number of long-term survivors achieved by the different treatments [13].

In vitro experiments

Cell cultures
Cultures of L1210 and Walker carcinosarcoma 256 were obtained from Prof K Harrap and Dr P Goddard, from the section of Drug Development of the Institute of Cancer Research, UK. EAT cells were obtained from the Department of Experimental Chemotherapy of Theagenion Anticancer Hospital. The L1210 cells were grown in RPMI 1640 medium, containing 10% calf serum, streptomycin, penicillin with 42 mM HEPES. The Walker cells were grown in Dulbecco's MEM + 10% horse serum and antibiotics. EAT cells were grown in medium 199, supplemented with 10% calf serum, antibiotics and 42 mM HEPES.

Treatment with nitrosoureas
The experiments were carried out in a cell suspension with 1 x 10^6 cells/ml. The compounds were dissolved in DMSO and then culture medium was added. The final concentration of DMSO, not more than 0.5%, had no cytotoxic effect in our testing system. The incubation time was 60 min at a concentration of 5 μg/ml at 37°C.

Incorporation studies
DNA, RNA and protein synthesis was determined after 30 min incubation of 10^6 cells and 1 μCi of radioactive precursors (5-^{3}H-thymidine, 5-^{3}H-uridine, L-4,5-^{3}H-leucine) in 1 ml final volume of the media mentioned above. The cell suspension was placed on Whatman filters no 41 and the wet filters were soaked in 5% cold TCA for 10 min. In the case of estimation of radioactive RNA, the filters were divided into two identical groups. The first group was left in 5% TCA at room temperature and the other was extracted in 5% TCA at 90°C for 20 min [14]. All the filters of the three precursors were further washed twice in 5% TCA, twice in 96% alcohol, once in a mixture of ether:ethanol (1:1) and once in ether. After drying, the filters were placed in scintillation fluid and the radioactivity was determined. The true radioactivity was obtained as the difference of counts in the total cold TCA and the radioactivity remaining after hot TCA extraction.

In vitro SCE assay

Lymphocyte cultures were prepared by adding 4 drops of heparinized whole blood from normal subjects to 4 ml of chromosome medium (IA Gibco). The cultures were incubated for 72 h at 37°C. Metaphases were collected during the last 2 h after colchicine treatment of 0.3 μg/ml. Treatment with the chemicals started 18 h after the initiation of the culture. For SCE demonstration, 5-bromodeoxyuridine (BdUrd) at 4 μg/ml was added 24 h after initiation of the culture. All cultures were kept in dark to minimize photolysis of BdUrd. Air-dried preparations were made and stained by the fluorescence plus Giemsa procedure. The preparations were scored for cells in their 1st, 2nd and 3rd and subsequent divisions with criteria previously described [10]. Student's t-test was performed to determine whether any values deviated significantly from the controls. The χ^2-square test was used for the cell kinetic comparisons. SCEs and PRIs were scored after the slides had been coded.

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