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Synthesis and biological activity of canavanine hydrazide derivatives

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Summary. The canavanine derivatives L-canavanine hydrazide (CH), L-canavanine-bis-(2-chloroethyl)hydrazide (CBCH) and L-canavanine phenylhydrazide (CPH) were synthesized and evaluated for biological activity in microorganisms, plants and tumor cells using canavanine as a positive control. (1) In microbial systems, the compounds exerted activity, as assessed in 14 bacterial strains. The effect of canavanine was easily removed by equimolar concentrations of arginine or ornithine, while the effect of CBCH or CPH was abolished by 10-fold excess of arginine or 10- to 100-fold excess of ornithine. (2) In plants, the activity of CH and CBCH were relatively low, whereas the inhibitory potential of CPH was comparable or even superior to that of canavanine, resulting at 1 mM concentration in a nearly complete block of tomato cell growth, and reducing by up to 80% the length of radicles of cress, amaranth, cabbage and pumpkin. (3) In pumpkin seeds, CPH or canavanine induced the synthesis of four small heat shock proteins of hsp-17 family in the pH range of 6 to 7.5. The proteins exhibited in both cases a similar profile, but differed in the timing of their expression and/or accumulation. With canavanine, the highest hsp-17 expression was found after 48 h of drug treatment, while with CPH this maximum was shifted to 24 h. (4) CPH proved to be highly cytotoxic against Friend leukemia cells in culture, exceeding by one order of magnitude the cytotoxicity of canavanine. The effect of canavanine was completely removed in the presence of equimolar amounts of arginine, while a 20-fold excess of arginine failed to abolish the cytotoxicity of CPH. Thus, a proper hydrazide modification of canavanine may lead to a significant increase in its growth-inhibitory activity and to a change in the mode of action of the parent compound.

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Keywords: Amino acids – Canavanine hydrazide derivatives – Inhibitory activity – Microorganisms – Plants – Tumor cells – Heat-shock proteins

Abbreviations: 1D-/2D-PAGE: One/two-dimensional polyacrylamide gel chromatography; DIPEA: N-Ethyldiisopropylamine; DTE: Dithiothreitol; TBTU: 2-(1H-Benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; TFA: Trifluoroacetic acid; IEF: Isoelectric focusing; MIC: Minimal inhibitory concentration; PMFS: Phenylmethylsulfonyl fluoride; PVDF: Polyvinylidene fluoride; CAPS: 3-(Cyclohexylamino)propanesulfonic acid; SDS: Sodium dodecyl sulfate; HSP: Heat shock protein.

Introduction

L-Canavanine (Cav, L-amino-4-(guanidinooxy)butyric acid), a natural arginine (Arg) analog found in many leguminous plants, plays a seminal role in plant chemical defence against a variety of organisms. As a substrate of arginyl-tRNA synthetase, it is incorporated into proteins in place of Arg, producing thereby structurally aberrant proteins with impaired function (Rosenthal et al., 1989; Rosenthal, 1992). Cav is toxic against viruses (Robertson et al., 1984), microorganisms and insects (Rosenthal, 1988, 1992b), inhibits plant cell growth in culture (Mengoli et al., 1989), and causes growth retardation of radicles of several plants (Avila and DeGarcia, 1984; Miersch et al., 1988, 1992; Schwartz et al., 1997). A number of studies have demonstrated antitumor activity of Cav towards tumor cells in culture and experimental tumors in vivo. This non-protein amino acid was reported to be active against Walker carcinoma cells, human melanoma or pancreatic cancer cells (Kruse and McCoy, 1958; Mattei et al., 1992; Swaffar et al., 1994) as well as with in vivo L1210 murine leukemia and rat colon tumor (Green et al., 1980; Thomas et al., 1986). Synergistic antitumor effects upon combination of Cav with γ-radiation (Green and Ward, 1983) or 5-fluorouracil (Swaffar et al., 1995) have been demonstrated. Thus, the synthesis of Cav analogs appears to be a promising approach in the development of new metabolic inhibitors with antimicrobial, pesticidal or antitumor activity.

Previously we obtained a number of Cav derivatives selectively blocked at the guanidino function and/or with a modified carboxylic group (Pajpanova et al., 1989, 1997, 1998). We found that canavanine amide and canavanine methyl ester inhibited significantly the growth of cultured tomato cells and radicles of cress and amaranth (Pajpanova et al., 1992).

Rosenthal et al. (1995) have reported insecticidal activity of some new Cav derivatives, and recent studies have revealed that several ester derivatives of Cav (especially n-butyl- and n-octyl-Cav) exerted enhanced growth-inhibitory activity towards cultured pancreatic carcinoma cells compared to Cav (Na Phuket et al., 1997).

In the present work, we report the synthesis and the inhibitory effects of a series of canavanine hydrazide derivatives on the growth of microorganisms and plants. The most active compound, canavanine phenylhydrazide (CPH), was further examined for its effect on protein synthesis in plants. In addition,

the cytotoxicity of CPH in Friend leukemia (FL) cells in culture was contrasted with that of Cav, using a cell-growth and macromolecular (DNA, RNA and protein) synthesis assays. Preliminary reports on these investigations appeared elsewhere (Pajpanova et al., 1992; Miersch et al., 1997).

Results

Synthesis of canavanine hydrazide analogs

The hydrazide derivatives of Cav (Fig. 1) were prepared by a condensation reaction of the corresponding di-protected compound Nα,N$_G$-Di-tert.-butyloxycarbonyl-L-canavanine (Boc-Cav(Boc)-OH) with the appropriate hydrazide in the presence of DIPEA, using TBTU reagent. The condensation method we have applied afforded high purity products and a column chromatography purification was not required. Moreover, it should be noted that under the mild conditions of the synthesis, undesirable side processes, including racemization, did not take place. Cleavage of the protecting Boc-group was achieved by ethyl acetate saturated anhydrous HCI (1.5N HCl/EtOAC), or TFA/anisole (9:1) in 95–98% yields.

The purity of the new Cav derivatives was evaluated by TLC and HPLC. They had the calculated elemental composition and their structures were confirmed by ^{1}H-NMR and mass spectrometry. The yields, mass and ^{1}H NMR spectral data as well as some other physicochemical parameters are given in "Materials and methods".

An increased lipophilic character of the hydrazides compared to Cav was registered by the greater R_f values on silica gel chromatography in a variety of solvent systems.

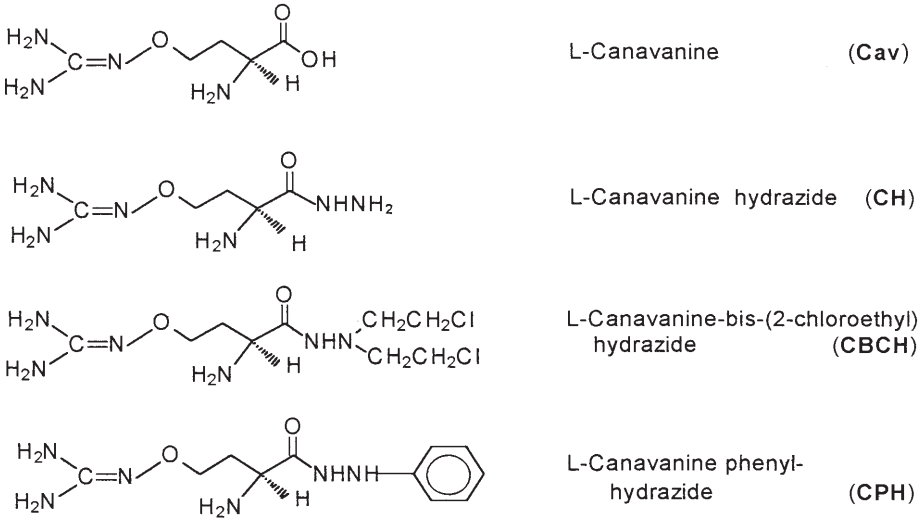

Fig. 1. Structure of canavanine hydrazide derivatives

The hydrazide derivatives were quite stable in aqueous solutions. The decomposition of CPH after five days incubation with plant seedlings at 37°C was less than 2%, as assessed by TLC and UV-spectroscopy.

Antimicrobial activity

The antimicrobial effect of the Cav hydrazide derivatives on 14 bacterial strains was assessed (Table 1). Cav, CBCH, and to lower extent CH, showed activity against *Bacillus subtilis*, *B. cereus* and *Proteus vulgaris*, while CPH inhibited the growth of *Klebsiella pneumoniae* strains and *Staphylococcus aureus*. So, compared to Cav, CBCH and CH, CPH was more active against Gram positive bacteria and less active towards Gram negative strains. The effect of Cav was reversed by equimolar amount of Arg, while 10-fold higher molar concentrations of Arg were required to abolish the effect of CBCH.

The effect of CPH was also abolished by a 10-fold excess of Arg. However, if the concentration of the CPH exceeded 4-times its MIC, no de-inhibition took place, even at 100-fold Arg excess. On the other hand, reversal of the effect of CBCH and Cav occurred even when they were present in 16 times their MIC concentrations.

Ornithine (Orn) was also found to remove the effect of all three analogs, but at 10-fold excess to CBCH and at 100-fold excess to CPH. Again, the effect of CPH could not be abolished at 4-fold its MIC or higher, while the concentration of CBCH did not influence the de-inhibition by Orn.

Table 1. Antibacterial activity of canavanine hydrazide derivatives

Microorganisms	Minimum inhibitory concentration (mM)			
	Cav	CH	CBCH	CPH
Klebsiella pneum. 450	>1.6	n.d.	>1.6	0.125
Kl. pneumoniae 52940	>1.6	n.d.	>1.6	0.25
Escherichia coli DI	>1.6	n.d.	>1.6	>2.0
E. coli DH-Amp	0.4	n.d.	>1.6	>2.0
E. coli 387	>1.6	>2.5	>1.6	>2.0
E. coli pR 55	>1.6	>5.0	>1.6	2.0
Proteus vulgaris	0.07–0.03	0.25–0.13	0.1	>2.0
Pseudomonas aeruginosa	>1.6	n.d.	0.4	>2.0
P. putida	n.d.	n.d.	n.d.	2.0
Bacillus cereus	0.63–0.31	1.8–0.63	0.4	2.0
B. subtilis	0.02–0.01	0.14–0.07	0.02	0.5
Sarcina lutea	>2.5	>2.5	–	1.0
Staphylococcus aureus 209 P	n.d.	n.d.	n.d.	0.25
Candida albicans	>5.0	>5.0	–	>2.0

n.d. not determined; – no inhibitory effect.

Effect on tomato cell growth

The inhibitory effects of Cav derivatives (at 1 mM concentration) on the growth of cultured tomato cells, up to 7 days of cultivation, are presented in Table 2. The most active compound proved to be CPH (57% and 91% growth inhibition on 3rd and 7th day, respectively), followed by Cav, whereas CH practically did not influence cell growth. CBCH exerted also inhibitory activity (34% inhibition), the effect being, unlike CPH and Cav, non-lethal. Upon prolonged treatment with this compound, the cells recovered from the inhibition reaching a dry mass value near to that of controls.

Effect on the growth of plant seedlings

The influence of Cav derivatives on the growth of plants was further studied using plant seedlings of cress, amaranth, cabbage and pumpkin. As shown in Fig. 2, Cav was most inhibitory against these model systems reducing at 1 mM concentration the length of radicles by 80–95%. CPH was little less effective (50–80% inhibition) exceeding, however, significantly the growth-inhibitory activity of CBCH and CH. Interestingly, towards cabbage CBCH did not exhibit any activity. Resuming the above results, the following orders of decreasing activity could be given: CPH > Cav > > CBCH > CH (tomato), Cav > CPH > CBCH > CH (cress), Cav=CPH > CBCH > CH (amaranth), Cav > CPH > > CBCH (cabbage), Cav > CPH (pumpkin).

In vitro cytotoxicity against murine tumor cells

The cytotoxicity of Cav and CPH in FL cells in DMEM (Dulbecco's modified Eagle medium) was examined using a cell-growth and macromolecular synthesis assays. In Table 3, the growth-inhibitory effects of 24, 48 and 72 h incubation of FL cells with these compounds are presented. The concentrations of Cav reducing the number of living cells by 50% ranged from 1.7 to 6 mM, while those of CPH ranged from 0.23 to 0.44 mM. A similar inhibition profile was observed when testing the effect of these compounds on macromolecular synthesis in FL cells. Cav concentrations, required to reduce by 50% [^{3}H]thymidine, [^{14}C]uridine or [^{14}C]leucine incorporation into DNA, RNA or protein, respectively, amounted from 1 to 1.45 mM, while those for CPH were 0.135–0.195 mM. Thus, against FL cells in culture CPH proved to be one order of magnitude more cytotoxic than Cav.

This was further confirmed by competition experiments. The growth-inhibitory effect of Cav on FL cells was completely removed in the presence of equimolar amounts of Arg, while a 20-fold excess of Arg failed to abolish the cytotoxicity of CPH. FL cells exposed to 5 mM Cav in DMEM for 24 h (62% inhibition of cell growth), recovered completely when the Cav-containing medium was removed and replaced with fresh medium. At the same time, the cells failed to recover upon CPH exposure (at concentrations higher than 0.5 mM), indicating irreversible cell damage.

Table 2. Influence of canavanine hydrazide derivatives on the growth of cultured tomato cells.
Cell suspensions of *Lycopersicon esculentum* were incubated with 1 mM Cav hydrazide derivatives at 28°C in the dark. On the days indicated, the optical turbidity at 570 nm was measured to follow cell growth

Compound	Time of cultivation (days)							
	2		**3**		**4**		**7**	
	DM[1] mg/ml	Inhibition %	DM[1] mg/ml	Inhibition %	DM[1] mg/ml	Inhibition %	DM[1] mg/ml	Inhibition %
Control	9.1 ± 1.38	0	13.0 ± 0.24	0	26.8 ± 7.90	0	38.3 ± 4.82	0
Cav	6.4 ± 0.25	30	5.8 ± 0.41	57	9.3 ± 0.25	69	7.7 ± 0.96	80
CH	8.8 ± 0.90	4	12.2 ± 2.03	9	23.8 ± 2.95	11	45.0 ± 4.64	−17[2]
CPH	3.4 ± 0.26	63	4.9 ± 0.19	63	6.8 ± 0.32	75	3.4 ± 0.16	91
CBCH	7.3 ± 0.67	20	11.6 ± 2.48	11	17.7 ± 4.9	34	33.9 ± 1.08	11

[1] DM, dry mass; [2] Growth stimulation.
Mean values + SD of quadruplicate determinations in three independent experiments are given.

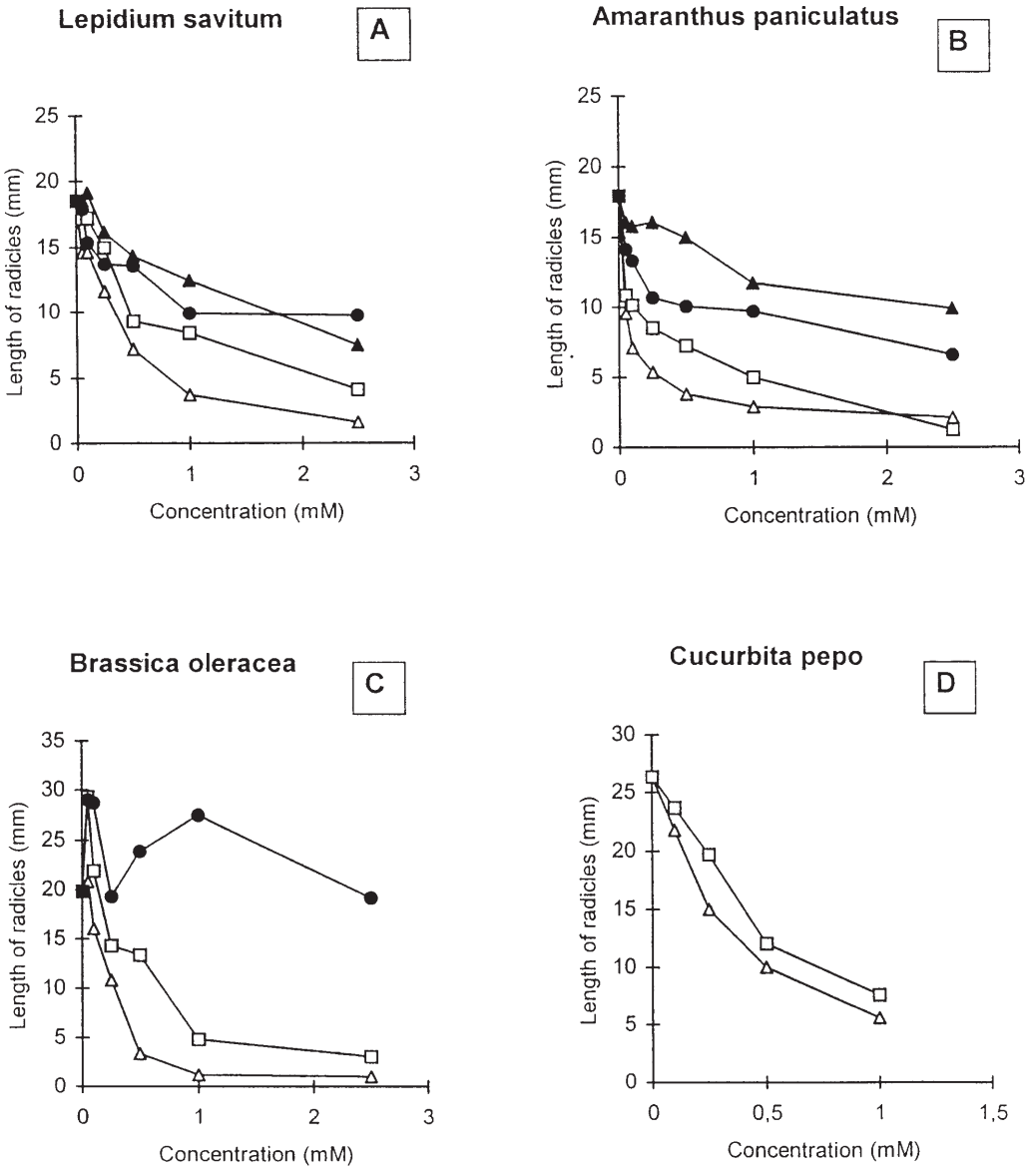

Fig. 2. Inhibitory activity of Cav (△), CPH (□), CBCH (•) and CH (▲)on the growth of radicles of cress (**A**), amaranth (**B**), cabbage (**C**) and pumpkin (**D**). The points are means of 24 to 136 measurements and are statistically significant by F-test (1%) and by chi^2-test (1%)

Effects on protein synthesis

The effect of the most active analog CPH on protein synthesis in plants was further examined and compared with that upon Cav or heat treatment. Cytosolic proteins of germinating seeds of *Cucurbita pepo,* treated with heat (1 h at 35°C, and for 2 h at 40°C), 1 mM Cav or 1 mM CPH, were analysed by IEF, SDS-PAGE (Fig. 3), 2-D PAGE (Fig. 4), and immunoblotting (Figs. 5, 6, 7). The comparison of the protein patterns of control and CPH-treated plants

Table 3. In vitro cytotoxicity of Cav and CPH in friend leukemia cells

Compound	Cell growth inhibition[1]			Macromolecular synthesis inhibition[2]		
	IC_{50}, mM[3]			IC_{50}, mM[4]		
	24 h	48 h	72 h	DNA	RNA	Protein
Cav	>6	5.5	1.7	1.45	2.88	1.0
CPH	0.44	0.315	0.23	0.195	0.15	0.135

[1] Exponentially growing cells were incubated in culture medium for 24, 48 and 72 h at 37°C with varying amounts of the derivatives and counted thereafter hemocytometrically. The number of dead cells was determined by trypan blue exclusion.
[2] The FL cells were incubated with varying amounts of the compounds for 24 h at 37°C. [^{3}H]Thymidine, [^{14}C]uridine or [^{3}H]leucine was added and the incubation continued for an additional 1 h to monitor precursor incorporation into DNA, RNA or protein, respectively. Aliquots of cells were pippeted on filter paper discs, washed with TCA and acid-insoluble radioactivity was determined by scintillation counting.
[3] Drug concentration that reduces the number of living cells by 50%.
[4] Drug concentration that reduces precursor incorporation into acid-insoluble material by 50%.
Values are means of quadruplicate determinations in at least two independent experiments.

revealed striking differences (Fig. 3), especially at four protein areas (Fig. 4). A clear induction of heat-shock protein (hsp) synthesis was registered after Cav- or CPH-exposure, as demonstrated by the immunological studies using anti-hsp-70 and anti-hsp-17 antibodies (Figs. 5 and 7). CPH induced the synthesis of hsp-17 (class II) protein family, analogous to that upon heat (Fig. 5 A) or Cav treatment (Fig. 7 A). On the other hand, the enhancement of hsp-70 protein synthesis was not clearly registered because of the constitutive hsp-70 expression. Analyzing further the protein species of hsp-17 family induced in pumpkin, we found that at least four major proteins in the range of pH 6 to 7.5 were labeled by anti-hsp-17 of tomato (Fig. 6 A–C). The pattern of hsp-17, induced by CPH, was similar to that upon Cav treatment. However, the timing of hsp-17 induction by the compounds tested revealed significant differences (Fig. 7 A, B) The highest expression and/or accumulation of hsp-17 was found after 48 h of Cav exposure (Fig. 7 B), whereas with CPH this maximum was shifted to 24 h (Fig. 7 C). This shift reflects most probably differences in the toxicological properties of these compounds.

Discussion

The literature data on the synthesis and biological activity of canavanine derivatives are scarce. Only a few derivatives have been described so far, among them a methyl ester (Kitagawa et al., 1932), some cyclic compounds (Kitagawa et al., 1937; Rickert et al., 1968), an Nα-benzoyl and an amide derivatives (Nakatsu, 1959). Recently, several Cav analogs (mainly aliphatic

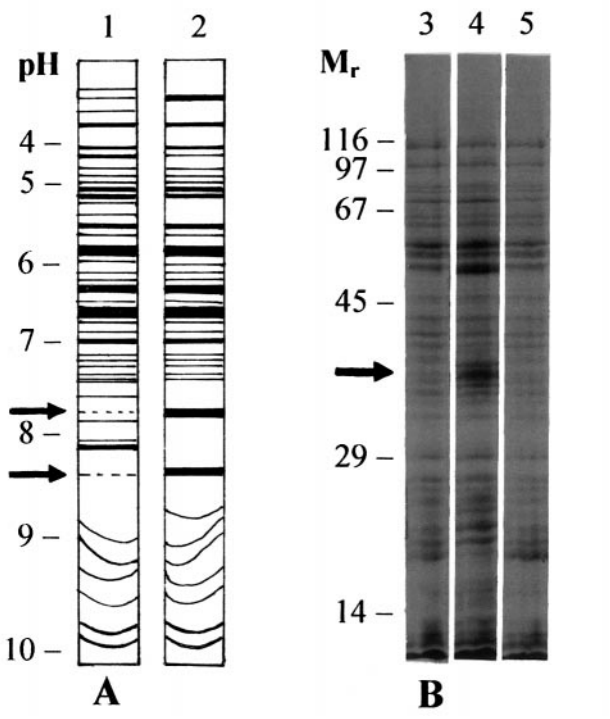

Fig. 3. Influence of canavanine phenylhydrazide on protein synthesis of pumpkin seedlings (*Cucurbita pepo L.*). Buffer-soluble proteins of seedlings, cultivated for 4 days, were separated by IEF (**A** lane *1* = control, lane *2* = canavanine phenylhydrazide, CPH, both 80μg protein) and 12% SDS-PAGE (**B** lane *3* = CPH, 20μg protein, lane *4* = 40μg protein, lane *5* = control, 40μg protein). Protein pattern of control and CPH-treated seedlings show striking differences in the range of pH 3–4 and about 8 (see arrows in lane *1*,*2*) as well as in molecular weight of 30–40 kDa (see arrow in lane 3–5). The immobilized pH-gradient (**A**) was calibrated using protein standards with the pI-values of 9.4, ribonuclease; 8.2, myoglobin, whale; 7.2, myoglobin, horse; 5.9, conalbumin; 5.1, β-lactoglobulin; 4.6, albumin, bovine; 3.3, amyloglucosidase. The SDS-gel (**B**) was calibrated using protein standards with the relative molar masses (kDa): 14, α-lactalbumin; 29, carbonic anhydrase, bovine erythrocytes; 45, albumin, egg; 67, albumin, bovine; 97, phosphorylase B; 116, β-galactosidase, E. coli; and 204, myosine

esters) have been developed and some of them shown to exhibit insecticidal properties or enhanced growth-inhibitory activity towards cultured tumor cells (Rosenthal et al., 1995; Na Phuket et al., 1997).

Following our current interest in application of non-protein amino acids in preparative peptide synthesis, we have synthesized several amino acid derivatives as structural analogs of asparagine, lysine (Videnov et al., 1993) and arginine (Pajpanova et al., 1997). In addition, amides and hydrazides of various protein amino acids as well as peptides containing modified amino acids are being extensively studied for biological activity. Our efforts in this field have led in recent years to the development of a number of Cav analogs which might be useful both as metabolic inhibitors, and in the modern peptide chemistry for the synthesis of Cav modified biologically active peptides

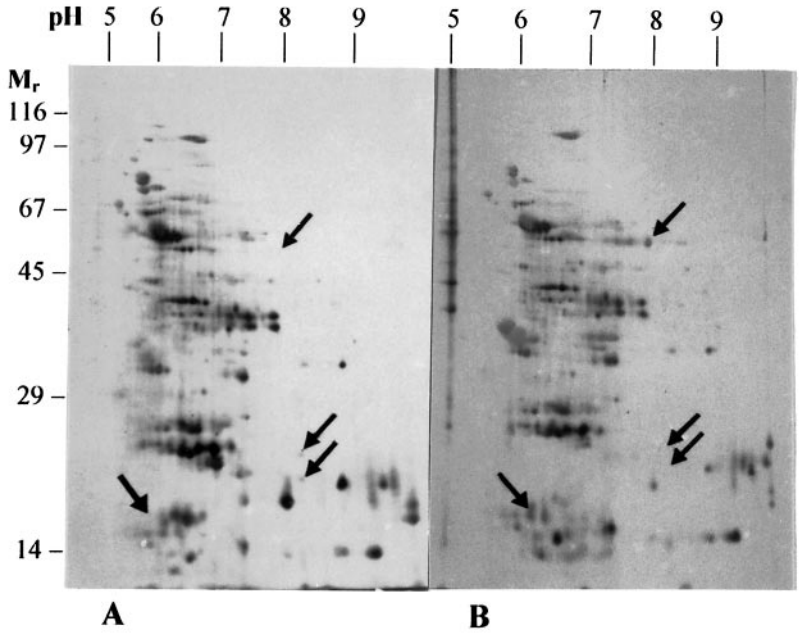

Fig. 4. Two-dimensional separation of proteins of pumpkin seedlings (*Cucurbita pepo* L.) influenced by canavanine phenylhydrazide. The separation of 80 μg of buffer-soluble proteins was carried out by IEF using an immobilized pH-gradient in the first dimension, and by 12% SDS-PAGE in the second dimension. Protein patterns of control (**A**), and CPH-treated (**B**) plants. Some striking differences in protein spots are indicated by arrows. Gels were calibrated using protein standards as described in the legend of Fig. 3 B

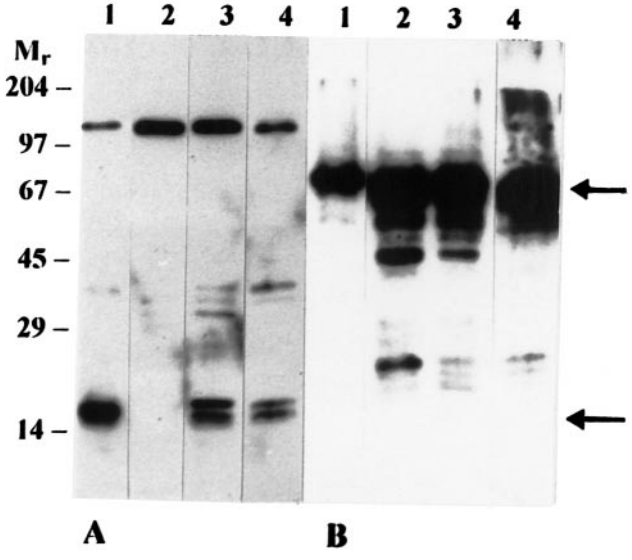

Fig. 5. Influence of canavanine phenylhydrazide on 17 and 70 kDa heat shock protein synthesis. Buffer-soluble proteins of seedlings were extracted, 15 μg of protein were subjected to each lane and separated by 12% SDS-PAGE. After blotting of proteins onto PVDF-membranes, the hsp 17 (**A**) and hsp 70 (**B**) were detected with anti-hsp 17 and anti-hsp 70 respectively of tomato (see Material and methods). The figures illustrate the serological results with 2.5 μg of tomato hsp (17 kDa, lane **A** *1*; 70 kDa, lane **B** *1*), and each of 15 μg protein of pumpkin seedlings cultivated under 23°C (control, lane **A** *2*, **B** *2*), 40°C (lane **A** *3*, **B** *3*), and 23°C with CPH-treatment (lane **A** *4*, **B** *4*). Gels were calibrated using protein standards as described in legend of Fig. 3

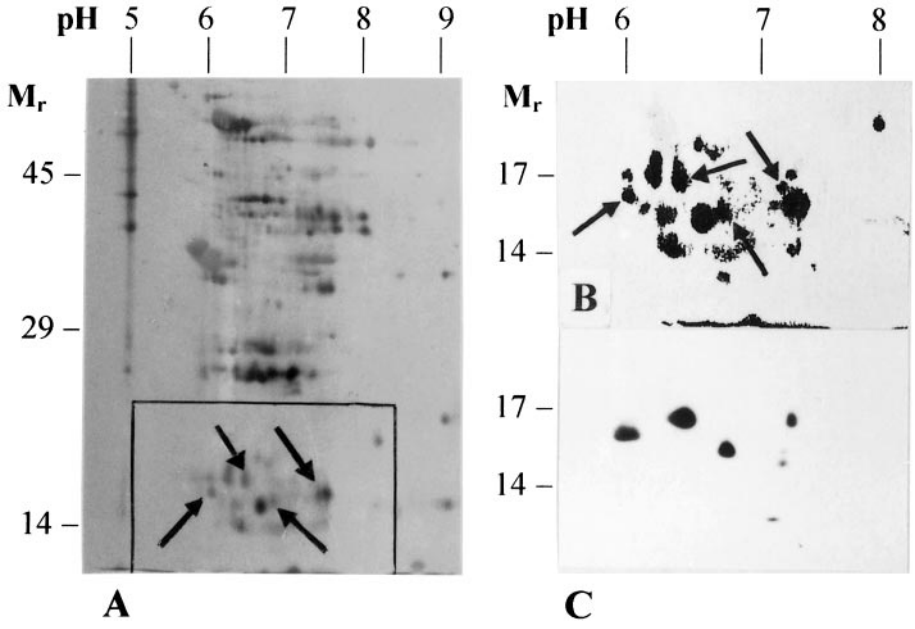

Fig. 6. Two-dimensional separation and Western Blot analysis of 17 kDa heat shock proteins in pumpkin seedlings after treatment with canavanine phenylhydrazide. Proteins were separated two-dimensionally by IEF and 12% SDS-PAGE as described in Fig. 4. The silver-stained proteins of CPH-treated seedlings (box in **A**) were analyzed by a personal computer using the Image Master 2 D program and magnified (**B**). The characterization of four protein spots of hsp 17 was carried out by Western blot analysis using anti-hsp 17 of tomato (**C**). The areas of hsp 17 are indicated by arrows in **A** and **B**. In control plants no proteins of hsp 17 familiy were detected. Gels were calibrated using protein standards as described in the legend of Fig. 3, and a standard hsp 17 of tomato

(Pajpanova et al., 1989, 1992, 1997, 1998). We were further interested in the study of the effect of new Cav analogs modified at the carboxylic group. Here we synthesized and evaluated the biological activity of a series of Cav hydrazide derivatives using a broad spectrum of test systems (microorganisms, plants and tumor cells).

CH, CBCH or CPH showed activity against several bacterial strains. The de-inhibition studies with Arg or Orn revealed interesting properties. The effect of CBCH and CPH on bacteria was abolished by 10-fold excess of Arg, and by 10-fold (CBCH) or 100-fold (CPH) excess of Orn. On the other hand, we found that the effect of CPH could not be reversed even by 100-fold excess of Arg or Orn, when the CPH concentration was 4-fold higher than the MIC, while CBCH, even at 16-fold excess of its MIC was completely competed by either Arg or Orn. Although both CPH and CBCH had equal MIC for *K. pneumoniae,* and CBCH was slightly more active against other strains of microorganisms, the lack of de-inhibition of the CPH effect suggest that it probably acts via a different mode.

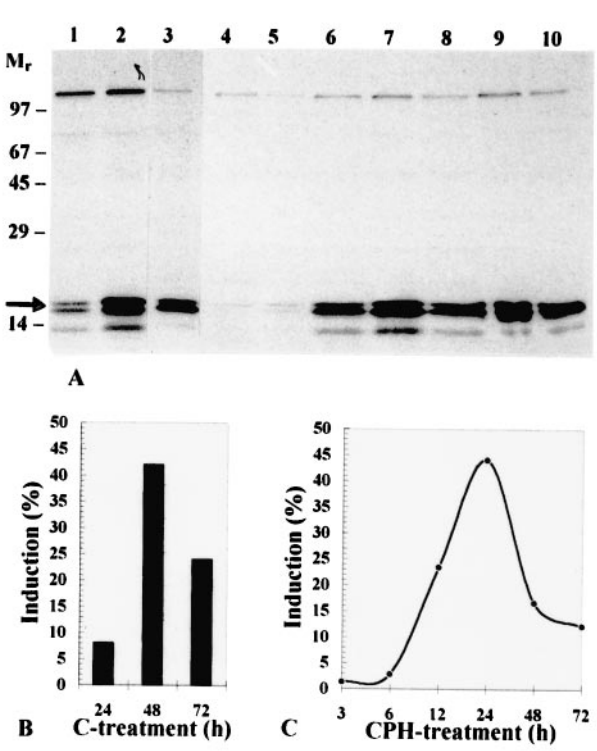

Fig. 7. Time-dependent synthesis of 17 kDa heat shock proteins induced by canavanine and canavanine phenylhydrazide in pumpkin seedlings. Cytosolic proteins of control (water-grown plants,) canavanine- and CPH-treated seedlings were extracted, 20 μg of protein were subjected to each lane and separated by 12% SDS-PAGE. After Western blot analysis using anti hsp 17 of tomato cells and Western Light luminescence technique, the proteins were visualized by gradation of black spots on the film (**A**) and quantified (**B**, **C**). **A** Synthesis of hsp 17 as indicated by arrow in seedlings grown at 23°C and treated with canavanine for 24, 48 and 72 h (lanes *1–3*) or with CPH for 3, 6, 12, 24, 48, and 72 h (lanes *4–9*). The presence of hsp 17 in control plants cultivated at 40°C is demonstrated in lane *10*. The protein pattern of control plants grown at 23°C is shown in Fig. 5 A (lane *2*). The exposed film was scanned and the amount of each fraction was quantified by a computer at each lane, SD = 6%. **B** Quantitation of lanes *1–3* (*C* canavanine); **C** Quantitation of lanes *4–9*. The gels were calibrated using protein standards corresponding to Fig. 3

In plants, the activity of CH and CBCH were relatively low, while the growth-inhibitory potential of CPH was comparable or even superior to that of Cav, resulting in a nearly complete block of tomato cell growth, and up to 80% reduction of radicle growth of cress, amaranth, cabbage and pumpkin. Inhibitory effects of Cav on the growth of cultered callus from *Nicotiana tabacum*, or zea mays roots have been previously reported (Avila and DeGarcia, 1984; Schwartz et al., 1997), the inhibition been readily removed by Arg.

The study of small heat shock protein (shsp) induction in plants exposed to Cav or CPH provided further evidence on mode of action of these analogs. sHsp play a particular role in cellular protection against heat shock, oxidative

stress, a variety of toxic chemicals and other inducers of apoptosis (Arrigo, 1998). In plants, they accumulate up to 1% of total leaf or root cell protein in response to heat, are quite stable following stress (with half-lives of 30–50 h) and appear to be critical for survival and recovery (DeRocher et al., 1991; Hsieh et al., 1992 Waters et al., 1996). We found that at least four major cytosolic shsp-17 (class II) were strongly expressed in pumpkin seeds upon Cav or CPH treatment. The proteins exhibited in both cases a similar profile, but differed in the timing of expression and/or accumulation, reflecting probably the different modes of action of Cav and CPH. The greater lipophilicity and cytotoxicity of CPH obviously results in an earlier shsp induction, compared to Cav, which should be incorporated into proteins to injure the cells.

In FL cells in culture, CPH exceeded by one order of magnitude the cytotoxicity of Cav, as assessed by cell-growth and macromolecular synthesis assays. This finding may have significant implications. *In vivo* as well as *in vitro,* Cav is effectively competed by Arg, and this attenuates the toxic effect of Cav. When Arg is present in the medium, a very high concentration of Cav would be needed to overcome this competition, and to exert activity. Swaffar et al. (1994) have reported a IC_{50} level of 2.5 mM Cav against human pancreatic cancer cell line in a medium with 0.4 mM Arg. Our tests with FL cells confirmed this observation. Likewise, an *in vivo* antitumor effect with Cav could be achieved only at very high doses under continuing drug infusion (Green et al., 1980) which might have unpredictable toxicological consequences. On the other hand, CPH proved to be highly cytotoxic against FL cells in culture in the presence of 0.4 mM Arg, and this effect was not removed upon a 20-fold increase of Arg concentrations. Thus, our findings suggest that a proper hydrazide modification of the carboxylic group of Cav may lead to a significant increase in the growth-inhibitory activity, and to a change in the mode of action of the parent compound. This encourages the development of antitumor agents from such canavanine derivatives.

Materials and methods

Canavanine sulfate was purchased from Sigma. The amino acid derivative Boc-Cav(Boc)-OH was prepared according to Pajpanova et al. (1997). The synthesis of L-canavanine hydrazide was principally carried out as previously reported (Pajpanova et al., 1989, 1997). All other reagents were AR grade products.

Synthesis of canavanine hydrazide derivatives

Nα,N_G-Di-tert.-Butyloxycarbonyl-L-canavanine phenylhydrazide, Boc-Cav(Boc)-NHNHC$_6$H$_5$. The Boc-Cav(Boc)-OH (376 mg, 1 mmol) was dissolved in 1 ml DMF and treated at 0°C with TBTU (353 mg, 1.1 mmol) in DMF (2 ml). After stirring at 0°C for 5 min, a pre-cooled solution of 1.2 mmol phenylhydrazine in DMF (1 ml) containing 2 mmol DIPEA was added and the mixture stirred at room temperature. After stirring for 4 hours, the DMF was evaporated *in vacuo* to a syrup, which was dissolved in 50 ml ethyl acetate (EtOAc), and washed subsequently with 5% citric acid, water, 10% $NaHCO_3$ and water. The organic phase was dried over Na_2SO_4, and evaporated *in vacuo* to a smaller volume. The product was precipitated by addition of petroleum ether. Yield 0.42 g (90%); m.p. 90°C; homogeneous (TLC system A and C); $[\alpha]_D^{23} = +25.1$ (c = 1, DMF); IR (KBr):

$\tilde{\nu}$ = 3,420 cm^{-1} (N-H), 3,290, 2,934, 2,840, 1,717 (C = N), 1,650 (C = O), 1,529, 1,359, 710 (CH-C_6H_5); ^{1}H NMR ($CDCl_3$): δ = 1.40 (s, 18 H, 6 CH_3 – Boc), 2.03 (m, 2 H, β CH_2), 3.75 (m, 2 H, γ CH_2), 3.9 (m, 1 H, α CH), 6.15 (s br, 3 H, guanidino-NH), 7.10 (d, 1 H, NH); MS-ES; *m/z* (%): 467 [M^+] – $C_{21}H_{34}N_60_6$ (466.537).

L-canavanine phenylhydrazide (CPH). To an ice-cooled solution of 1.5 N HCI in EtOAc, 466 mg of the protected compound (1 mmol) was added. The solution was stirred for 2 hours at room temperature and precipitated with cold diethyl ether. The resulting crude product was crystallized from absolute ethanol/diethyl ether, washed with diethyl ether and dried *in vacuo* over P_2O_5. Yield 260 mg (98%); m.p. 226°C (dec.); homogeneous (TLC system A and C); $[\alpha]_D^{23}$ = +26.6 (c = 1, DMF); MS-ES; *m/z* (%): 267.2 [M^+] – $C_{11}H_{18}N_60_2$ (266.363).

Nα,N_G-Di-tert.-Butyloxycarbonyl-L-Canavanine-bis-(2-chloroethyl)hydrazide, Boc-Cav(Boc)-NHN(CH_2)$_4$$CI_2$. According to the procedure described above, 376 mg Boc-Cav(Boc)-OH (1 mmol) were treated with bis-(2-chloroethyl)hydrazine (232 mg, 1.2 mmol) for 4 hours at room temperature. The product was crystallized from EtOAC/petroleum ether. Yield 510 mg (96%); m.p. 59–62°C; homogeneous (TLC system A and C); $[\alpha]_D^{23}$ = +20.2 (c = 1, DMF); IR (KBr): $\tilde{\nu}$ = 3,420 cm^{-1} (N-H), 3,290, 2,934, 2,840, 1,717 (C = N), 1,650 (C = O), 1,529, 1,359, 745 (C-CI); ^{1}H NMR ($CDCl_3$): δ = 1.40 (s, 18 H, 6 CH_3 – Boc), 2.03 (m, 2 H, β CH_2), 3.75 (m, 2 H, γ CH_2), 3.9 (m, 1 H, α CH), 6.15 (s br, 3 H, guanidino-NH), 7.10 (d, 1 H, NH); MS-ES; *m/z* (%): 516 [M^+] – $C_{19}H_{36}N_60_6CI_{62}$ (515.436).

L-Canavanine-bis-(2-chloroethyl)hydrazide (CBCH). Compound Boc-Cav(Boc)-NHN(CH_2)$_4$$CI_2$ (515 mg, 1 mmol) was treated with 1.5 M HCl in EtOAc as described for CPH. Yield 298 mg (95%); m.p. 94–97°C (dec.); homogeneous (TLC system A and C); $[\alpha]_D^{23}$ = +18.0 (c = 1, DMF); MS-ES; *m/z* (%): 315.2 [M + H^+] – $C_9H_{20}N_60_2CI_2$ (314.202).

Analyses

The purity of the compounds was checked by TLC as well as by reversed-phase HPLC. Their structures have been confirmed by ^{1}H NMR and by electrospray mass spectrometry.

In order to check the chemical stability of the derivatives, they were incubated in aqueous solution for up to 72 h at room temperature and their UV-spectra recorded. Likewise, CPH was incubated up to 5 days in the presence of plant seedlings at 37°C and analyzed periodically by TLC.

TLC was carried out on silica gel 60F_{254} plates (Merck) using of the following solvent systems: A = n-butanol : acetic acid : water (4 : 1 : 5); B = chloroform : methanol: water (80 : 30 : 5); C = chloroform : methanol : NH_3 (2 : 2 : 0.5). The spots were visualized with either UV, ninhydrin or a pentacyanoammonioferrate (PCAF) reagent. Melting points were determined on a Kofler melting point apparatus. Optical rotation was measured with a Perkin-Elmer Model 141 polarimeter. IR spectra were recorded on Specord-71-IR (Zeiss) or Bruker 113V spectrophotometers. UV spectra were recorded on Beckman spectrophotometer. ^{1}H NMR spectra were recorded on Bruker WM-250 and Avance DRX-250 (250 MHz) spectrometers. Mass spectra were recorded on an Fissons-Triple-Quadrupol-ES mass spectrometer.

Determination of the antimicrobial activity

The following bacterial strains were used: *Escherichia coli*, strains DI, DH-Amp, 387 and pR 55, *Proteus vulgaris*, *Bacillus cereus; B. subtilis, Klebsiella pneumoniae*, strains 450 and 52940, *Staphylococcus aureus*, strain 209 P, *Pseudomonas aeruginosa, P. putida, Sarcina lutea and Candida albicans.*

The antimicrobial activity was determined by the broth dilution method (Ashdown, 1988). Briefly, the canavanine derivatives were dissolved in nutrient media (Nutrient broth Difco for bacteria, or Sabouraud for *C. albicans*) at 2-fold the desired test concentrations, and 0.1 ml aliquots of each dilution were dispensed in Nunc sterile flatbottom 96-well plates. Inoculation was carried out with 0.1 ml bacterial suspension, prepared by

diluting overnight cultures of the respective strains with fresh nutrient broth to a density of 1×10^6 cfu/ml or, with suspensions of *C. albicans*, with a density of 1×10^5 cfu/ml. The plates were incubated at 37°C for 24 h (bacteria), or at 30°C for 48 h (*C. albicans*), and then the MIC values were recorded, taken as the lowest concentration of the compound at which no visually determinable growth occurred.

For the de-inhibition of bacterial growth, aliquots (0.05 ml) of CPH or CBCH solutions in M9 nutrient medium at final concentrations as high as 16, 8, 4, 2 or 1 times the MIC of the compounds for the strain were dispensed in the wells. To these solutions 0.05 ml of Arg or Orn were added to give a molar ratio of amino acid to inhibitor of 100, 10, 1 or 0.1, respectively. Thereafter, inoculation with 0.1 ml bacterial suspension in M9 medium was carried out. The presence of bacterial growth was determined visually upon 24 h incubation at 37°C.

Inhibitory effect on tomato cell growth

Cell suspension of *Lycopersicon esculentum* MILL. cv Lukullus was cultured at 23°–25°C on rotatory shakers at 150 rpm as described previously (Grancharov et al., 1985). The mid-log phase cells were incubated with different concentrations of the inhibitor for 24, 48, 72, 96 and 168 h. Thereafter, the optical turbidity at 570 nm was measured to monitor cell-growth inhibition. The average value of quadruplicate determinations in at least three independent experiments was calculated.

Plant seedling inhibition studies

Seeds of cress (*Lepidium sativum* L.), amaranth (*Amaranthus paniculatus* L.), cabbage (*Brassica oleracea* L.) and pumpkin (*Cucurbita pepo* L.) were used to follow the influence of canavanine derivatives on radicle growth. The seedlings were incubated for 96 h at 28°C and 80% humidity in the dark, and the length of the radicles was determined as previously described (Miersch, 1992). The mean of 24–136 measurements in at least two separate experiments was calculated.

Plant cytosolic proteins

Seeds of pumpkin (*Cucurbita pepo* L. cv. Riesenmelonen, Samen-Mauser Quedlinburg GmbH) were soaked in water for 6 h, changing the water 3–4 times. The seeds were washed two times with bi-distilled water and placed on a filter paper for two days in a glass tray (20 × 30 cm) saturated with water. Then, each 5 seeds were placed on a filter paper disk in Petri dish (9 cm of diameter), soaked with 6 ml bi-distilled water (control), or 6 ml of 1 mM Cav or CPH, respectively. The plant material was cultivated in a plant culture chamber (Rumed, Rubarth, Hannover) in 5 parallels for 2 additional days, harvested and stored at −80°C until used. Each sample (0.7 g cotyledons) was homogenized for 2 min in 2–5 ml of ice-cold 25 mM Tris-HCl, pH 7.5, containing 3 mM EDTA, 10 mM DTE, and 0.2% (w/v) PMFS. The homogenate was centrifuged at 6000 × g for 15 min.

Protein determination

Five microlitres of the supernatant were mixed with 1 ml of 5% trichloroacetic acid (TCA) to precipitate the protein overnight at 4°C. The precipitate was washed with 1% TCA and water, and the protein amount determined according to Lowry et al. (1951) with bovine serum albumin as a standard.

Sample preparation

Generally, proteins were precipitated by a pre-chilled acetone at −20°C overnight, centrifuged at 7000 × g for 15 min, and dissolved as described below. For SDS-PAGE, 100 μg

of protein were dissolved in 100 μl Sigma-buffer (60 mM Tris-HCl, pH 6.8, 10% (w/v) glycerol, 2% (w/v) SDS, 5% (v/v) mercaptoethanol). Before electrophoresis, the samples were boiled for 2 min at 100°C, and thereafter 2 μl of a 50% (w/v) bromphenol blue solution were added. In case of 2-D PAGE, the proteins were dissolved in lysis buffer (8 M urea, 2% (w/v) Nonidet NP 40, 5% (v/v) mercaptoethanol, 0.8% (v/v) Servalyte 3–10 (Serva, Heidelberg)), according to Görg (1991).

SDS-PAGE

One-dimensional SDS-polyacrylamide gel electrophoresis was carried out in a 5% (w/v) stacking gel and 12% (w/v) separating gel, or in a 10–14% (w/v) gradient gel in the buffer systems of Laemmli (1970) or O'Farrel (1975). The proteins were visualized by staining with Coomassie Brilliant Blue R-250 (Serva, Heidelberg) or silver, according to the method of Heueskoven and Dernick (1988). The standard protein mixtures are described in figure legends. Unless otherwise noted, the low molecular weight proteins are directed to the anode.

2-D PAGE

For separation in the first dimension, 80 μg protein, dissolved in 40 μl lysis-buffer, were subjected to isoelectric focusing (IEF) for 45–50 kVh on an immobilized pH gradient of 3–10 (Immobiline, Pharmacia-Biotech, Freiburg) using the Multiphor Electrophoresis-System (Pharmacia-Biotech, Freiburg) described by Werner et al. (1990). After IEF, the proteins were separated by SDS-PAGE in the second dimension as described above. The gels were fixed and stained with Coomassie Brilliant Blue or silver.

Isoelectric focusing

To determine the pI-values of unknown proteins, 10 μg per lane were subjected to Servalyt Precotes (Serva, Heidelberg, pH-range of 3–10). For calibration of IEF-gels, a pH-standard protein mixture 9 (Serva, Heidelberg) was used: amyloglucosidase (3.3), ferritin (4.4), bovine albumin (4.6), β-lactoglobulin (5.1), conalbumin (5.9), horse myoglobulin (8.2), ribonuclease (9.4), and cytochrome C (10.5).

Western blot

Proteins were transblotted from gels using CAPS-buffer, pH 11, on Immobilon PVDF membranes for 1.5 h at 250 mA, and for 0.5 h at 350 mA. The immunoblotting procedure was carried out as described by Neumann et al. (1987) using polyclonal anti-hsp-17 and anti-hsp-70-antibodies. The Western Light immunodetection system (Tropix/Applied Biosystems, Weiterstadt) was applied. Spots on films were quantified using a densitometer CD 60 (Desaga, Heidelberg, Germany).

In vitro cytotoxicity

Cell-growth assay. FL cells (clone F 4N) were grown in DMEM, supplemented with 10% calf serum, under 5% CO_2 atmosphere at 37°C. The cultures were passed every day at a concentration of 5×10^5 cells/ml. Exponentially growing cells were incubated in triplicate with increasing concentrations of the inhibitors for 24, 48 and 72 h, and counted thereafter hemocytometrically. The number of dead cells was determined by staining with trypan blue. The mean of two independent experiments was calculated. The 50% inhibitory dose (IC_{50}) was defined as drug concentration required to reduce the number of living cells by 50%.

In order to determine if the inhibitory effect of Cav or CPH was reversed by Arg, FL cells were exposed in triplicate to different concentrations of the inhibitors for 24, 48 or

72 h in a medium containing 3 mM, 6 mM and 12 mM Arg (5-fold, 10-fold or 20-fold more Arg, respectively, than that of DMEM), and counted as above.

In order to examine the cell recovery upon drug treatment, cells were exposed to the inhibitors in DMEM for 24 h, Cav or CPH-containing medium was removed by centrifugation (800 × g for 5 min) and replaced with fresh medium. The cells were incubated for additional 24, 48 and 72 h, and counted as above.

Macromolecular synthesis assay. The macromolecular synthesis determination was essentially carried out as previously described (Grancharov et al., 1988). FL cells at a concentration of 0.5×10^6 cells/ml were incubated with incremental concentrations of inhibitors. Thereafter, [^{3}H]thymidine (0.02 MBq), [^{14}C]uridine (0.02 MBq) or [^{14}C]leucine (0.04 MBq) was added and the cells were incubated for an additional 1 h to follow precursor incorporation into DNA, RNA or proteins, respectively. Aliquots of cell suspension were pipetted on Whatman filter paper discs, the acid-soluble radioactivity was extracted with cold TCA acid (5%), and the incorporated activity measured by scintillation counting. The mean of quadruplicate determinations in at least two independent experiments was calculated.

Statistics

The data were analyzed statistically by ANOVA.

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