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Growth inhibition of HepG2 cell line by canavanine, norcanavanine and their hydrazide derivatives

Kaloyan Georgiev[1], Tatyana Dzimbova*[2], Ivan Iliev[3], Anelia Balacheva[2], Roumyana Detcheva[2], Martin Schmid[4] and Tamara Pajpanova[2]

[1]*Medical University of Varna "Prof. Dr. Paraskev Stoyanov", Faculty of Pharmacy, 55 Marin Drinov str., Varna 9002, Bulgaria*
[2]*Institute of Molecular Biology "Roumen Tsanev", Bulgarian Academy of Sciences, Acad. G. Bonchev str., block 21, Sofia 1113, Bulgaria*
[3]*Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences, Acad. G. Bonchev Str., block 25, Sofia 1113, Bulgaria*
[4]*Karl-Franzens University of Graz, Institute of Pharmaceutical Science, Department of Pharmaceutical Chemistry, Univ. Platz 1, Graz 8010, Austria*

ABSTRACT

A number of hydrazide derivatives of the non-protein amino acid L-canavanine (Cav) and its structural analogue L-norcanavanine (NCav), have been synthesized and their growth-inhibitory effects evaluated in cultured non-tumor 3T3 cells and tumor cell line HepG2 by the MTT assay. The Cav derivatives (Cav-CONHNC$_6$H$_5$, Cav-CONHN(CH$_2$CH$_2$Cl)$_2$, and Cav-CONHNH$_2$) and NCav derivatives (NCav-CONHNHC$_6$H$_5$ and NCav-CONHN(CH$_2$CH$_2$Cl)$_2$ exhibited higher cell growth inhibitory effects on both cell lines compared to their parent compound. These effects were considerably higher on the tumor cells HepG2 in comparison to the non-tumor 3T3 cells. Introduction of the hydrazide group constitutes an effective structural modification, which significantly amplifies the growth inhibitory properties of the parent compound against tumor cells HepG2. These results confirm our previous conclusion that a proper hydrazide modification of the carboxylic group of Cav and its analogues may lead to a significant increase in the inhibitory effect of the compounds on the growth of tumor cells. On the other hand, NCav derivatives showed higher cytotoxicity on the HepG2 cells in comparison with the respective canavanine analogues. Evidently the length of the carbon side chain influences the cytotoxicity of the compounds also.

Keywords: Cytotoxic activity· Canavanine· Norcanavanine· Hydrazide deivatives· Human tumour cell lines
Abbreviations *Cav - canavanine; NCav - norcanavanine; Can – canaline, 2-amino-4-(aminooxy)butyric acid; NCan – norcanaline; NsArg – norsulfoarginine; NHNH(CH$_2$CH$_2$Cl)$_2$ - bis-2(chloroethyl)hydrazine; EtOAc - ethyl acetate; TBTU - O-(Benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium tetrafluoroborate; NMM - N-Methylmorpholine; DIPEA - N,N-Diisopropylethylamine; Boc - Di-tert-butyl dicarbonate; Z - Carboxybenzyl*

INTRODUCTION

L-Canavanine (Cav) [L-2-amino-4-(guanidinooxy)butyric acid], a nonprotein amino acid found in certain leguminous plants [1], is a structural analogue of L-arginine in which the terminal methylene group is replaced by an oxygen atom [2]. As an analogue and antimetabolite of arginine, canavanine is highly toxic to a wide range of organisms including bacteria, fungi, yeast, algae, plants, insects, and mammals [3, 4].

Since the structural similarity of Cav to Arg is remarkable, it can effectively compete with arginine for arginyl tRNA synthase and for incorporation into cellular proteins [2]. It is important to note that at physiological pH less than half

of the L-canavanine molecules will be protonated and able to successfully compete with L-arginine for access to the active site of arginyl-tRNA synthetase [5, 6]. In addition, Cav is an inhibitor of inducible nitric oxide synthase [7, 8]. L-Canavanine has been reported to possess growth retardation activity toward tumor cells in culture and experimental tumors *in vivo* [9-12]. Synergic antitumor effects from a combination of L-canavanine with 5-fluorouracil or γ-irradiation have been demonstrated, indicating that L-canavanine may modulate the chemo- or radio-sensitivity of tumors [13, 14].

Although these effects suggest that the inhibitory mode of amino acid analogues toward tumors may vary depending on the analogue types and cell types [15-17], a primary mechanism underlying the antitumor activity of amino acid analogues, leading to cell damage, is thought to be their incorporation into cellular proteins in substitution for the intact forms and subsequent induction of structurally aberrant proteins with impaired function or degradation [18-20]. Since the oxyguanidino group of L-canavanine is electronically and structurally different than the guanidino group of L-arginine, there are several distinct differences between L-canavanine and L-arginine. When compared to L-arginine, the L-canavanine molecule is less basic, slightly longer and exists predominantly in the amino, rather than imino tautomeric form. Replacement of an L-arginine residue in a protein with L-canavanine can significantly affect key ionic interactions that determine the tertiary and quaternary structure of the protein. The erroneous incorporation of L-canavanine results in structural changes that alter protein function and form the basis of L-canavanine's anticancer activity [20, 21].

Chemotherapy employing antineoplastic drugs often relies on the difference of the mitotic rate between tumor and normal cells in order to confine its toxic effect to the tumor. In this regard, amino acid analogues have been simply considered to possess potency as chemotherapeutic agents because of their incorporation into cellular proteins, which results in an inhibitory effect on cell growth. This effect can be more significant in tumor cells than in normal cells. The inhibitory activity of amino acid analogues on the growth of tumor cells, and the underlying inhibitory mechanisms requisite for evaluating their potency as a chemotherapeutic agent, remain however largely unknown. In subsequent studies, L-canavanine preferentially inhibited the in vitro growth of transformed canine kidney epithelial cells when compared to normal canine kidney epithelial cells [21]. This observation led the authors to postulate that the basis for the selectivity of L-canavanine may be L-canavanine incorporation into cell surface proteins. Since transformed cells have fewer attachment proteins on their cell membrane, the structural changes resulting from L-canavanine incorporation into cell surface proteins in the transformed cells may have had a greater effect on the capacity of the cells to adhere than in the non-transformed cells.

The role of apoptotic cell death in the inhibitory activity of amino acid analogues against tumor cells is still poorly elucidated. Jang et al. have found that the cytotoxicity of L-canavanine toward human acute leukemia Jurkat T cells is attributable to induced apoptosis [22] *via* caspase-3 activation. Co-administration of canavanine significantly accelerated and enhanced apoptotic manifestations induced by arginine deprivation [23]. In view of this, it was interesting to design and synthesize unnatural amino acids containing a guanidino functionality (oxy- and sulfoguanidino), as structural analogues of arginine and canavanine [24-26]. Their effects on the growth of cultured tumor cells were evaluated [4, 27-29]. We found that modification of Cav at the carboxylic group selectively changed toxicity against tumour cell lines [4, 27].

In the present study, we examined the cytotoxic activities of L-Cav **1** and its shorter analogue L-NCav **2** (*Figure 1*), and their hydrazide derivatives [Cav-$CONHNH_2$ **11**, Cav-$CONHN(CH_2CH_2Cl)_2$ **13**, Cav-$CONHNC_6H_5$ **15**, NCav-$CONHNH_2$ **12**, NCav-$CONHN(CH_2CH_2Cl)_2$ **14,** and NCav-$CONHNC_6H_5$ **16**], on 3T3 and HepG2 cells.

MATERIALS AND METHODS

1.1. Synthesis

All chemicals were of analytical grade. All anhydrous solvents were obtained commercially (Fluka) and used directly. HPLC-grade acetonitrile and MeOH were purchased from Merck. Analytical TLC was performed on Merck silica gel (60F254) plates (0.25 mm) using of the following solvent systems: A: *V*(chloroform) :*V*(methanol):*V*(water)=80 : 30 :5; B: *V*(benzene) :*V*(acetone) :*V*(acetic acid)=100 : 50 : 2; C: *V*(chloroform) :*V*(methanol):*V*(acetic acid)=95 : 5 : 5. Visualization was done with either UV, ninhydrin or a chlorine tolidine reagent. Mass spectra were recorded on a Fissons-Triple Quadrupol-ES mass spectrometer. HPLC analyses were performed on Agilent Technologies HP 1100 and Waters 2695 LC instruments, using a Column: Lichrosphere® RP_8 (250 x 4,6 mm); mobile phase: 0.02 M K_2HPO_4 + 5% methanol. The fully protected amino acid analogues Boc-Cav(Boc_2)-OH and Boc-NCav(Boc_2)-OH were prepared as previously reported [4, 27].

General procedure for the preparation of the Cav and NCav hydrazide derivatives ***5-10***
Route a: To the solution of Boc-Cav(Boc)$_2$-OH **3** or Boc-NCav(Boc)$_2$-OH **4** (3 mM) in THF (5 ml) NMM (0.33 ml, 3 mM) was added, and the reaction mixture was cooled down to -10°C. Piv-Cl (0.37 ml, 3 mM) was added dropwise and after 10 minutes solution of hydrazine (Boc-NHNH$_2$, HCl.NH$_2$NHC$_6$H$_5$, or HCl.NH$_2$N(CH$_2$CH$_2$Cl)$_2$) (15 mM) and Et$_3$N (0.415 ml, 15 mM) in water (2 ml). Process completed after 1.5 at -10°C and the solvent was evaporated under reduced pressure. The residue was dissolved in CHCl$_3$ (10 ml) and was washed with water (2 x 10 ml). Organic layer was dried over Na$_2$SO$_4$, and CHCl$_3$ was evaporated. Pure products were obtained after column purification (silicagel, eluent,CH$_3$CN/H$_2$O, 4 : 1, *v/v*) and the respective yields of the compounds were 56-60%. Homogeneous (TLC system A and B).

Route b: Boc-Cav(Boc)$_2$-OH **4** or Boc-NCav(Boc)$_2$-OH **3** (3 mM) were dissolved in the mixture of DMF (1 ml) and DCM (2 ml) and treated at 0°C with TBTU (0.96 g, 3 mM). After stirring at 0°C for 5 min, a pre-cooled solution of 3.2 mM hydrazine in 1 ml DMF and DIPEA (0.52 ml, 3 mM) were added. Reaction mixture was stirred for 4 hours. Solvent was evaporated in vacuo, the residue was dissolved in CHCl$_3$ (10 ml) and washed with water (2 x 10 ml). Organic layer was dried over Na$_2$SO$_4$, and CHCl$_3$ was evaporated. Pure product was obtained after column purification (silicagel, eluent, CH$_3$CN/H$_2$O, 4:1, *v/v*) and the respective yields of the compounds were 81-86%. Homogeneous (TLC system A and B).

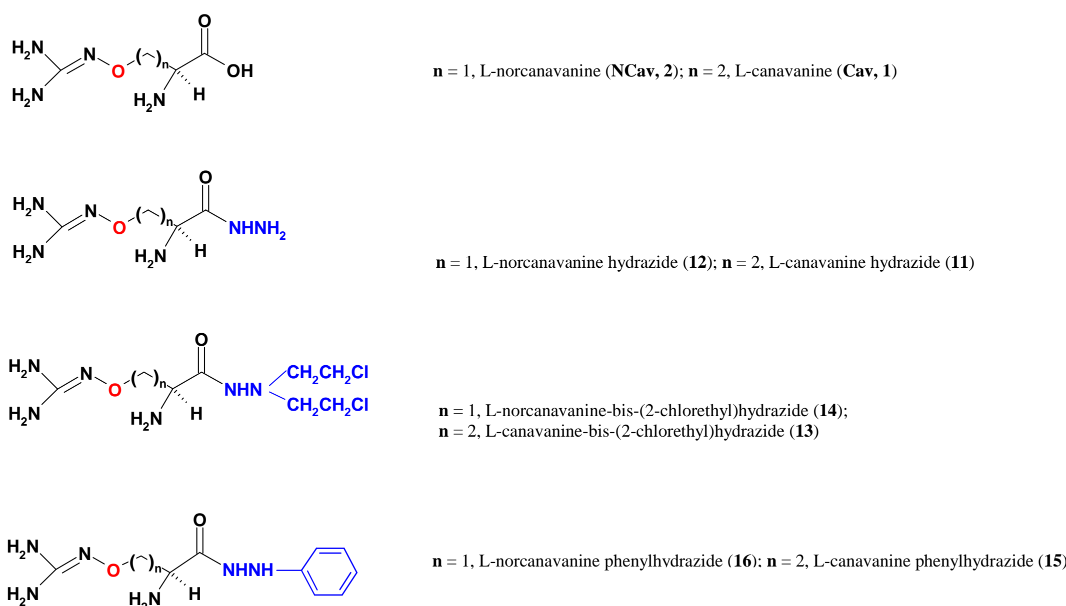


FIGURE 1: Structures of canavanine (n = 2) and nor-canavanine (n = 1) and their hydrazide derivatives

General procedure for deprotection of Boc-Protecting group: Protected analogues **5-10** (3 mM) were dissolved in EtOAc (1 ml) and 1.5M HCl/EtOAc (3 ml) was added. The reaction mixture was stirred at room temperature and solvent was evaporated in vacuo. Products were obtained after column purification (silicagel, eluent, CH$_3$CN/H$_2$O, 4:1, *v/v*).

11: MS-ES, *m/z*: 191.12 [MH$^+$] (190.12); ^{1}H-NMR (CDCl$_3$) *δ/ppm*: 8.56 (s, 2H, NH), 8.0 (s, H, NH), 5.11 (s, 2H, NH$_2$), 3.53 (m, 2H, CH$_2$), 3.37 (t, H, αCH), 2.0 (m, 3H, NH, NH$_2$), 1.96 (m, 2H, CH$_2$); ^{13}C-NMR (CDCl$_3$) *δ/ppm*: 170.4, 158.5, 66.7, 52.0, 34.2;

12: MS-ES, *m/z*: 176.10 [MH$^+$] (177.10); ^{1}H-NMR (CDCl$_3$) *δ/ppm*: 8.58 (s, 2H, NH), 8.2 (s, H, NH), 5.11 (s, 2H, NH$_2$), 4.16, 3.91 (m, 2H, CH$_2$), 3.65 (t, H, αCH), 2.1 (m, 3H, NH, NH$_2$); ^{13}C-NMR (CDCl$_3$) *δ/ppm*: 171.1, 158.7, 76.7, 53.7;

13: MS-ES, *m/z*: 267.15 [MH$^+$] (266.15); ^{1}H-NMR (CDCl$_3$) *δ/ppm*: 8.57 (s, 2H, NH), 7.9 (s, H, NH), 7.37 (m, 2H, Ar), 7.06 (m, 2H, Ar), 6.90 (m, H, Ar), 5.07 (s, 2H, NH$_2$), 4.0 (s, H, NH), 3.51 (m, 2H, CH$_2$), 3.35 (t, H, αCH), 2.4 (m, 3H, NH, NH$_2$), 1.98 (m, 2H, CH$_2$); ^{13}C-NMR (CDCl$_3$) *δ/ppm*: 170.2, 158.8, 149.0, 129.2, 122.8, 113.2, 66.3, 52.3, 34.0;

14: MS-ES, *m/z*: 253.14 [MH^+] (252.15); ^{1}H-NMR ($CDCl_3$) *δ/ppm*: 8.50 (s, 2H, NH), 7.8 (s, H, NH), 7.35 (m, 2H, Ar), 7.06 (m, 2H, Ar), 6.92 (m, H, Ar), 5.10 (s, 2H, NH_2),4.16, 3.91 (s, 2H, CH_2), 4.2 (s, H, NH), 3.65 (t, H, αCH), 2.1 (m, H, NH); ^{13}C-NMR ($CDCl_3$) *δ/ppm*: 171.3, 157.8, 149.3, 129.9, 121.8, 114.0, 76.3, 54.0;

15: MS-ES, *m/z*: 316.10 [MH^+] (314.10); ^{1}H-NMR ($CDCl_3$) *δ/ppm*: 8.60 (s, 2H, NH), 8.1 (s, H, NH), 5.10 (s, 2H, NH_2), 3.50 (m, 2H, CH_2), 3.40 (t, H, αCH), 3.50 (m, 4H, CH_2), 2.83 (m, 4H, CH_2), 1.90 (m, 2H, CH_2); ^{13}C-NMR ($CDCl_3$) *δ/ppm*: 170.4, 158.2, 66.7, 60.1, 52.1, 39.6, 34.2;

16: MS-ES, *m/z*: 302.09 [MH^+] (300.09); ^{1}H-NMR ($CDCl_3$) *δ/ppm*: 8.57 (s, 2H, NH), 8.3 (s, H, NH), 5.08 (s, 2H, NH_2), 4.16, 3.91 (m, 2H, CH_2), 3.62 (t, H, αCH), 3.52 (m, 4H, CH_2), 2.80 (m, 4H, CH_2); ^{13}C-NMR ($CDCl_3$) *δ/ppm*: 171.9, 157.7, 76.2, 59.8, 54.7, 39.4;

1.2. Cell cultures

The 3T3 (standard mouse embryonic fibroblast cell line) and HepG2 (human liver hepatocellular carcinoma cell line) cells were cultured in Dulbecco Modified Eagle's medium (DMEM) (Gibco, Austria) supplemented with 10% fetal bovine serum (Gibco, Austria), 100 U/ml penicillin (Lonza, Belgium) and 0.1 mg/ml streptomycin (Lonza, Belgium) under a humidified 5% CO_2 atmosphere at 37°C. Plastic flasks supplied by Greiner, Germany, were used to grow the cells. Cells were trypsinized using Trypsin-EDTA (FlowLab, Australia) when they reached approximately 80% confluence. The cells in the exponential phase of growth after treatment with Trypsin-EDTA were seeded into 96-well plates (Greiner, Germany) in a concentration of $2x10^4$ cells/well. 24 hours of incubation post seeding (under a humidified 5% CO_2 atmosphere at 37°C

1.3. Cytotoxicity assay

The cultivated cells were treated with amino acid analogues in a wide concentration range (2 - 0.015 mM). Untreated cells were used as controls. Empty wells were blank controls. Cytotoxicity was measured by colorimetric assay based on tetrasolium salt MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma Chemical Co.). The MTT assay is based on the protocol first described by Mossman [30]. In this assay, living cells reduce the yellow MTT to insoluble purple formazan crystals. Amino acid analogues were dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO in the samples did not affect the viability of the cells. The assay was performed 24 hours after treatment with the amino acid analogues. For this purpose, the MTT solution was prepared at 5 mg/ml in PBS and was filtered through a 0.2 μm filter. Then 1 ml of MTT solution was added to 15 ml DMEM and 100 μl of this solution was added into each well, including the cell free blank wells. Then the plates were further incubated for 3 hours to allow MTT to be metabolized and the supernatant liquid was removed. 100 μl/well DMSO/ethanol (1/1) was added. The plates were placed in a microtitre-plate shaker for 10 min at room temperature to mix thoroughly the purple formazan into the solvent. An ELIZA plate reader (TECAN, Sunrise TM, Grodig/Sazburg, Austria) was used for reading the results. Optical density (OD) was determined at a wavelength of 540 nm and a reference wavelength of 620 nm. Cell cytotoxicity determined by MTT assay was expressed as the percentage of dead cells:

% cytotoxicity = (1 - (OD sample – OD blank control)/(OD control - OD blank control)) x 100.

PrizmaPlot.4 (ANOVA-test) was used for statistical analysis. Graphics were created by GraphPad Prism4.

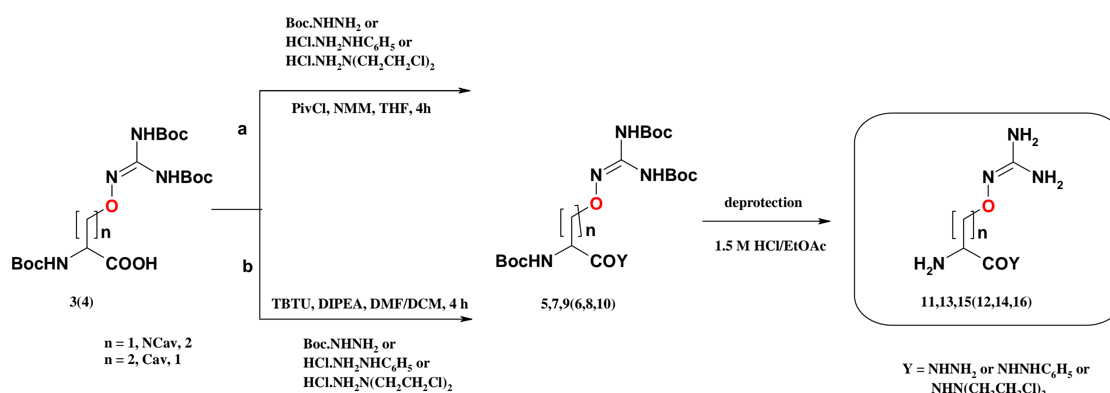
RESULTS

1.4. Chemistry

In an extension on our efforts to create new classes of amino acids containing oxy- and oxy- and sulfo-guanidino group, we report the preparation of several Cav and NCav hydrazides belonging to the canavanine series. As previously reported for the Cav [4, 25] and NCav [27], these amino acids ease converted into related hydrazides.

For the synthesis of the desired compounds, the sequence of reactions shown in synthetic scheme (*Scheme 1*) was followed. Fully protected Boc-Cav(Boc_2)-OH **3** and Boc-NCav(Boc_2)-OH **4** were the starting materials of our strategy. The synthetic approach was utilized to generate a series of hydrazide derivatives followed the *route **a*** by a condensation reaction with the appropriate hydrazine in the presence of NMM, using Piv-Cl, or *route **b***, reacting with the required hydrazine in the presence of DIPEA, using TBTU reagent.

The condensation methods we have applied afforded high purity products with good yields after column chromatography purification. Moreover, it should be noted that under the mild conditions of the synthesis, undesirable side processes, including racemization, did not take place.



SCHEME 1: Synthetic routes for the synthesis of Cav and NCav hydrazide derivatives

Cleavage of the protecting Boc-groups were achieved by ethyl acetate saturated anhydrous HCl (mixture of 1.5 M HCl and EtOAC).

In addition, the hydrazide derivatives were quite stable in aqueous solutions. The decomposition of Cav hydrazides **13, 15**, and NCav hydrazides **14, 16** after five days incubation with cell cultures at 37°C was less than 2%, as assessed by TLC and HPLC.

1.5. In vitro cytotoxicity of the compounds

The results of the cytotoxicity of canavanine and its derivatives Cav-CONHNC$_6$H$_5$ **15**, Cav-CONHN(CH$_2$CH$_2$Cl)$_2$ **13**, and Cav-CONHNH$_2$ **11** on 3T3 and HepG2 cells are shown in *Figure 2*. The cells were exposed for 24 h to different concentrations (ranging from 2 to 0.015 mM) of the compounds. As seen in *Figure 2* the treatment of the both cell lines resulted in a dose-dependent reduction of the number of the viable cells. Only the highest Cav concentrations (2 and 1mM) produced an inhibitory effect on the cell growth in HepG2 cells (28% and 14 %, respectively) (*Figure 2*). The Cav derivatives **11, 13** and **15** exhibited higher cell growth inhibitory effects on both cell lines compared to their parent compound. These effects were considerably higher on the tumor cells HepG2 where statistically reliable results were achieved for the most of the concentrations. Thus our results suggest that a proper hydrazide modification of the carboxylic group of Cav may lead to a significant increase in the cell growth inhibitory activity, which confirms and enlarges our previous conclusion [4] about the effect of Cav-CONHNC$_6$H$_5$ in Friend erythroleukemia cells.

The sensitivity of the cell lines was demonstrated by calculating the half maximal inhibitory concentrations (IC$_{50}$ values), shown in *Table 1*. The tumor cell line HepG2 is more sensitive to the cytotoxic effects of Cav-CONHNC$_6$H$_5$, Cav-CONHN(CH$_2$CH$_2$Cl)$_2$, and Cav-CONHNH$_2$.

When the exposure time of 3T3 and HepG2 cells was extended from 24 h to 48 and 72 h, the cell growth inhibitory effects of the compounds were more pronounced (results not shown).

The results of the cytotoxicity of NCav and its derivatives NCav-CONHNHC$_6$H$_5$ **16**, NCav-CONHN(CH$_2$CH$_2$Cl)$_2$ **14** and NCav-CONHNH$_2$ **12** on 3T3 and HepG2 cells are shown in *Figure 3*. The cells were exposed for 24 h to different concentrations (ranging from 2 to 0.015 mM) of the compounds. The treatment of the both cell lines resulted in a dose-dependent reduction of the number of viable cells. Only the highest NCav concentration (2 mM) manifested a cell growth inhibitory effect in both cell lines. In the 3T3 cells the inhibitory effect of **14** and **16** was, however insignificant. It was considerably higher on the tumor cells where statistically reliable results were achieved for most of the concentrations. The cell growth inhibitory effect of NCav-CONHNH$_2$ on the both cell lines was insignificant.

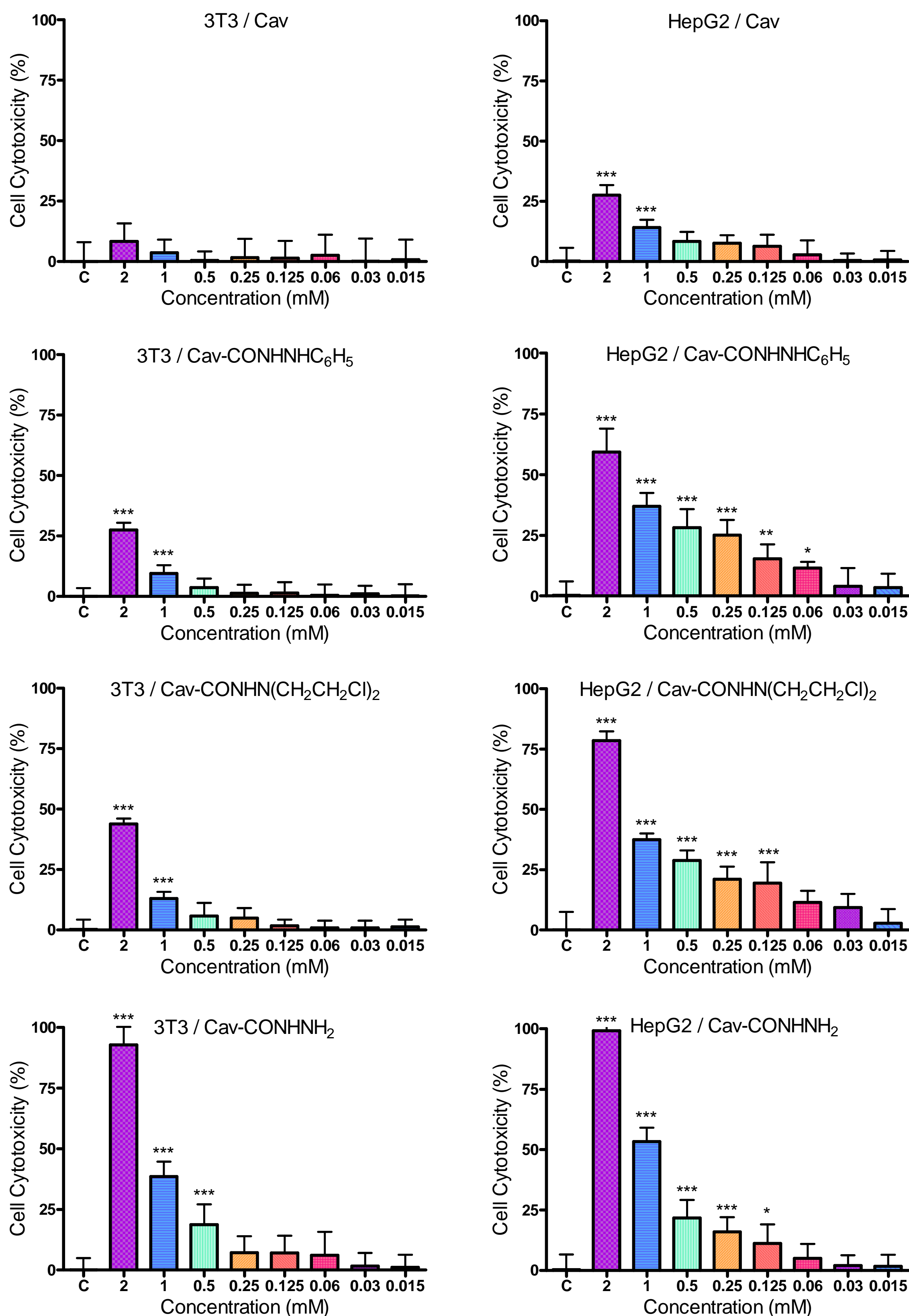


FIGURE 2: Effect of Cav and its analogues: Cav-CONHNHC$_6$H$_5$; Cav-CONHN(CH$_2$CH$_2$Cl)$_2$; Cav-CONHNH$_2$ on growth of 3T3 and HepG2 cells after 24 h treatment. Cell cytotoxicity determined by MTT assay is expressed as per cent of dead cells and presented as mean ± SD (n=6), **P<0.001, **P< 0.01, *P<0.05, ANOVA-test, versus the control group, C – control

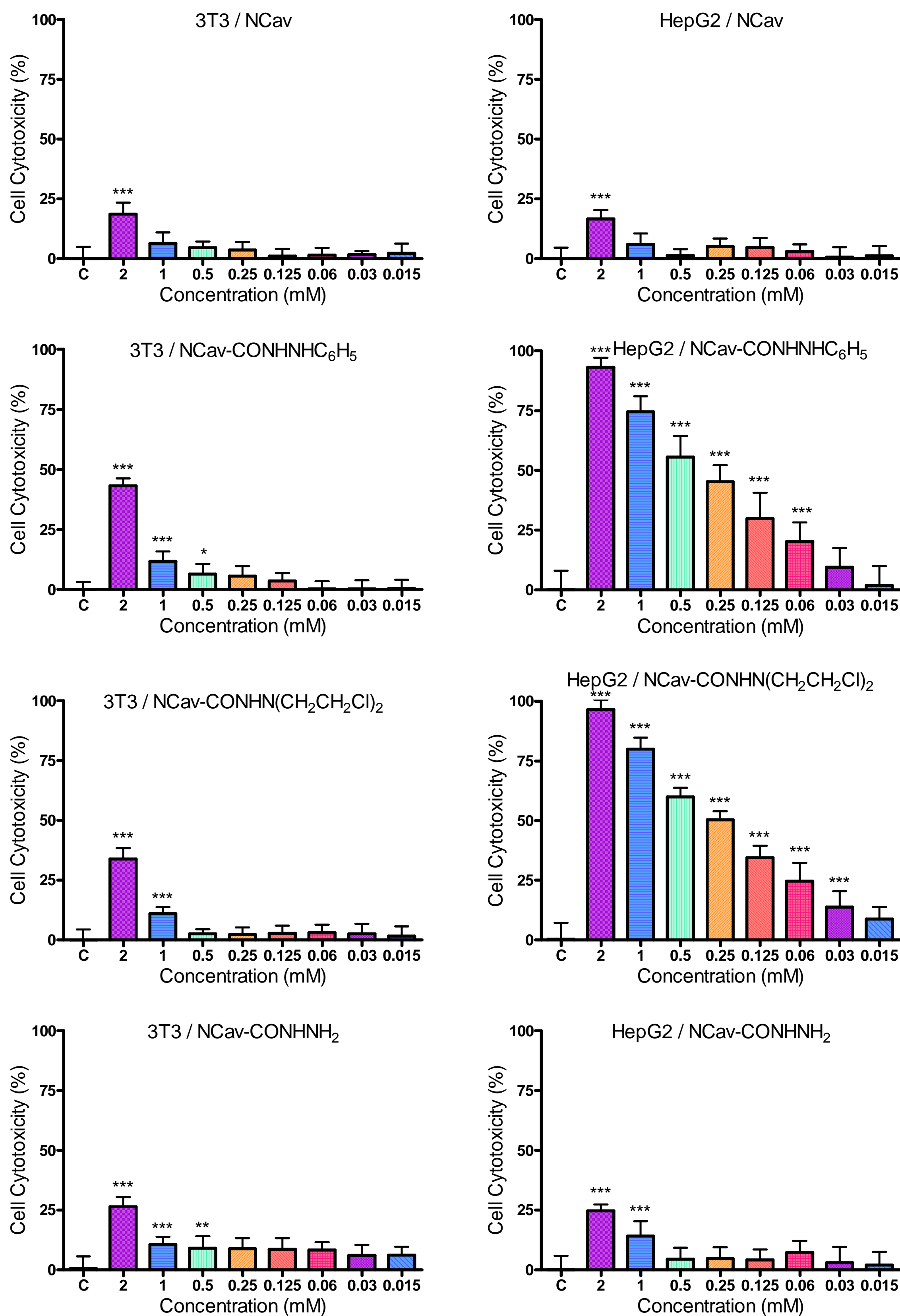


FIGURE 3: Effect of NCav and its analogues: NCav-CONHNHC$_6$H$_5$; NCav-CONHN(CH$_2$CH$_2$Cl)$_2$; NCav-CONHNH$_2$ on growth of 3T3 and HepG2 cells after 24 h treatment. Cell cytotoxicity determined by MTT assay is expressed as per cent of dead cells and presented as mean ± SD (n=6), **P<0.001, **P< 0.01, *P<0.05, ANOVA-test, versus the control group, C - control

As observed in the *Table 1* HepG2 (a tumour cell line) is more sensitive to the cytotoxic effects of the compounds **14** and **16** than is 3T3 (a non-tumor cell line). These two NCav-derivatives proved to be highly cytotoxic against HepG2, exceeding at least by an order of magnitude the cytotoxicity of their parent compound NCav. They showed higher cytotoxicity on the HepG2 cells in comparison with the respective canavanine analogues.

TABLE 1: Comparative cytotoxic activity of Cav, NCav and their analogues in 3T3 and HepG2 cells after 24 h treatment (MTT-dye reduction assay)

Compounds	Mean IC_{50} values (mM)*		
	Cell lines	3T3	HepG2
Cav		>2	>2
Cav-$CONHNHC_6H_5$		>2	1,642 ± 0,3082
Cav-$CONHN(CH_2CH_2Cl)_2$		>2	1,310 ± 0,0522
Cav-$CONHNH_2$		1,206 ± 0,0925	0,960 ± 0,0740
NCav		>2	>2
NCav-$CONHNHC_6H_5$		>2	0,417 ± 0,1244
NCav- $CONHN(CH_2CH_2Cl)_2$		>2	0,263 ± 0,0602
NCav-$CONHNH_2$		>2	>2

** Values are means ± SD (n=6)*

DISCUSSION

The results of the present study show that the substitution in the carboxylic group of Cav and NCav increases the cell growth inhibitory effects of the compounds – especially in the case of the tumor cell line HepG2. The same correlation was found previously in the case of sArg and its analogues [28]. An increase of the cell growth inhibitory effects was also observed with the modification of the carboxylic group in our experiments with canaline (Can) and its analogues (unpublished results). For example, IC_{50} of NCan-$CONHN(CH_2CH_2Cl)_2$ was found to be 0,84 +/- 0,094 mM after 24 h of incubation with HepG2 cells whereas IC_{50} of NCan was higher than 2 mM.

In *Table 2* we have summarized our previous results about the cytotoxicity of several amino acid analogues on Friend Leukemia cells, clone F4N. The growth-inhibitory effects of 24, 48 and 72 h incubation of the cells with the drugs are expressed by IC_{50} values. The cytotoxicities of L-Cav, Cav-$CONHNC_6H_5$, Cav-$CONHN(CH_2CH_2Cl)_2$, NCav, NCav-$CONHNHC_6H_5$, NsArg, NsArg-$CONHNHC_6H_5$, N_G-nitro-canavanine and N_G-nitro-canavanine methyl ester in F4N cells were examined using cell-growth assay. Clear differences in the cell growth were observed after 48 and 72 h of incubation. Cav-$CONHNC_6H_5$ proved to be highly cytotoxic against Friend Leukemia cells in culture, exceeding by one order of magnitude the cytotoxicity of canavanine [4]. NCav-$CONHNHC_6H_5$ proved to be 3 times more cytotoxic than NCav. Similar results were obtained for norsulfoarginine (NsArg) and its phenylhydrazide derivative NsArg-$CONHNHC_6H_5$ (*Table 2*). The second compound revealed a significantly higher cell growth-inhibitory effect. Methyl esterification in the carboxylic group of N_G-nitro-canavanine also caused an increase of its cytotoxic action. These results confirm our conclusion that the modification of the carboxylic group is important for the inhibitory effect of the compounds on the growth of tumor cells.

TABLE 2: Cytotoxicity of arginine analogues in Friend leukemia cells

Compounds	Cell growth inhibition[1] IC_{50} (mM)[2]			
	Time(h)	24	48	72
NCav*		>8	8	3.2
NCav-$CONHNHC_6H_5$*		>8	2.5	1.3
NsArg*		4	3.3	2.4
NsArg-$CONHNHC_6H_5$*		4.6	1.7	0.8
Cav**		>6	5.5	1.7
Cav-$CONHNHC_6H_5$**		0.44	0.315	0.23
Cav(NO_2)			4.25	2.16
Cav(NO_2)-OMe			1.82	1

[1]Exponentially growing cells were incubated in culture medium for 24, 48 and 72 h at 37°C with varying amounts of the compounds and counted thereafter hemocytometrically. The number of dead cells was determined by trypane blue exclusion.
[2]Drug concentration that reduces the number of living cells by 50%.
Values are means of triplicate determination in at least two independent experiments.
**Results from our previous research (Dzimbova et al. 2011; Dzimbova et al. 2012).*
***Results from our previous research (Miersh et al. 2000).*

The shorter canavanine analogue NCav and its derivatives **14** and **16** proved to be more cytotoxic against the tumor HepG2 cells in comparison to the respective canavanine analogues **13** and **15**. We conclude that the length of the side carbon chain influences the cytotoxicity of the compounds also.

The results of other studies suggest that L-canavanine may be useful as an antitumor agent. It has been shown that L-Cav can inhibit the proliferation of tumor cells *in vitro* and *in vivo* [4-5, 28]. In the present study we demonstrate that canavanine derivatives with a modification of the carboxylic group promise to be more potent as antitumor drugs than Cav. Little is known about the molecular mechanisms mediating these effects. Amino acids modified with alkylating groups may exert their effects simultaneously as alkylating agents and antimetabolites. The alkylating agents damage DNA attaching an alkyl group (C_nH_{2n+1}) to the guanine base of DNA. On the other hand arginine has numerous roles in the cellular metabolism that may influence the multistep process of cancer development [31]. Some animal and human tumors require arginine for growth. Whereas normal cells are able to synthesize arginine from citrulline, some human cancers, such as melanoma and hepatocellular carcinoma [32] are unable, because they do not express the enzyme arginosuccinate synthase.

Cav and its analogues we investigate in our study are antimetabolites of arginine and this may explain at least partly their cytotoxic action, especially on the tumor cell line HepG2. In our previous study [4] we showed that the cytotoxic effect of Cav on F4N cells was completely removed in the presence of equimolar amounts of arginine, whereas a 20-fold excess of arginine failed to abolish the cytotoxicity of Cav-CONHNC$_6$H$_5$. These results suggest to us that the modes of action of Cav-CONHNC$_6$H$_5$ and its parent compound are different.

It was demonstrated that the treatment of human lung adenocarcinoma A549 cells with Cav caused growth inhibition and G1 phase arrest [33]. The examination of Jang et al. [22] showed that the treatment of human Jurkat T cells with L-canavanine had induced apoptotic cell death via caspase-3 activation regulated by Bcl-2 or Bcl-xL.

The apoptotic and genotoxic activity of the most potent canavanine analogues, as well as their effects on the progression of cells through different phases of the cell cycle, will be a subject of our future investigations.

CONCLUSION

In summary, we have demonstrated that the synthesized hydrazide derivatives of canavanine and norcanavanine possessed cytotoxic activity against human liver hepatocellular carcinoma cell line. As expected, the hydrazides showed different activity depending on cell line and amount of the compound used. Pronounced cytotoxic effects against HepG2 cells were observed for compounds **13, 14, 15** and **16**. These results confirm the fact that the proper modification in the carboxylic group of the molecule is crucial for the cytotoxic activity.

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