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IL FARMACO 59 (2004) 271–277
IL FARMACO
www.elsevier.com/locate/farmac

Synthesis and evaluation of anti-apoptotic activity of L-carnitine cyclic analogues and amino acid derivatives

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Accepted 8 January 2004

Abstract

Two series of derivatives were synthesised. In one series (R)-4-hydroxy-2-pyrrolidinone was used as a mimic of cyclic L-carnitine analogue and in the second series 3-amino-2-piperidinone was used as a cyclic ornithine analogue. N-Benzyloxycarbonyl derivatives of some amino acids were also prepared. The newly synthesised compounds were tested for their ability to inhibit Fas-activated apoptosis of human Jurkatt T-cell line. The results confirm the previously described anti-apoptotic activity of carnitine and indicate new carnitine and amino acid analogues (**1, 3, 6, 7, 20**) that inhibit Fas-induced apoptosis.

Keywords: Carnitine; Carnitine cyclic analogues; Apoptosis

1. Introduction

Apoptosis, programmed cell death (PCD), is a physiological process that organisms use to eliminate unwanted cells, including tumour cells, and that is important in animal development, as well as in tissue homeostasis [1]. Moreover, improperly regulated apoptosis can lead to many pathological conditions. Inadequate or diminished apoptosis, associated with uncontrolled cell growth, results in cell accumulation and is a hallmark of cancer [2]. Excessive apoptosis leads to organ failure, alteration of homeostatic control and degenerative disorders, including some neuronal diseases [3]. In fact, apoptosis has been implicated in the aetiology of several human diseases, such as Alzheimer's [4], AIDS [5] and ischemic injury [6,7]. Clearly, the possibility of modulating, inducting or inhibiting apoptosis, by pharmacological agents necessitates generating and identifying new agents with potential anti-apoptosis activity. A number of studies and clinical trials have shown that L-carnitine has therapeutic effects under these conditions [8-10] which suggests that it may be acting as an anti-apoptotic agent [11-15]. In particular, it has been reported that carnitine inhibits caspase activation and Fas-induced apoptosis thus resulting in the survival of T lymphocytes [15].

As a part of our investigations aimed at discovering and developing new apoptosis-modulating compounds and within a project concerning new L-carnitine analogues, two series of derivatives have been synthesised. In one series, (R)-4-hydroxy-2-pyrrolidinone was used as a mimic of cyclic L-carnitine analogue; in a second series 3-amino-2-piperidinone was used as a cyclic ornithine analogue. Moreover, due to the structural similarity between carnitine and some amino acids (AA), such as glutamic acid, glutamine and asparagine, N-benzyloxycarbonyl derivatives of some amino acids were also prepared to test the hypothesis that benzyloxycarbonyl-group (Z) plays a role in the modulation of apoptosis [16].

All of the newly synthesised compounds were tested for their ability to inhibit Fas-activated apoptosis of human Jurkatt T-cell line. The preliminary results confirm the previously described anti-apoptotic activity of carnitine and also indicate new carnitine and AA analogues that inhibit Fas-induced apoptosis.

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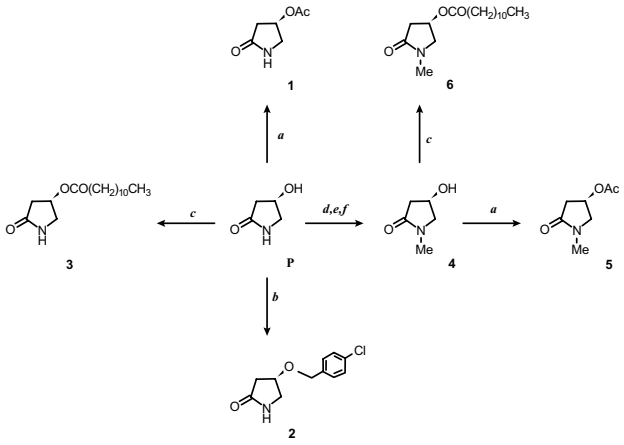
doi:10.1016/j.farmac.2004.01.004

2. Chemistry

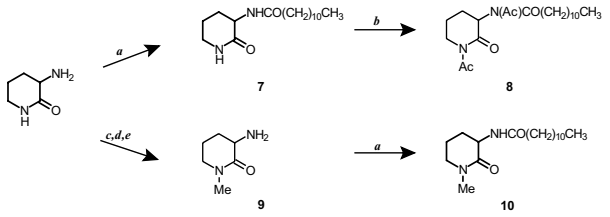
Starting from (R)-4-hydroxy-2-pyrrolidinone (**P**), (R)-4-acetoxy-2-pyrrolidinone (**1**) [17] was obtained by acylation, (R)-4-[(4-chlorobenzyl)oxy]-2-pyrrolidinone (**2**) was prepared by alkylation with 4-chlorobenzyl chloride, and (R)-4-lauroyloxy-2-pyrrolidinone (**3**) was synthesised by condensation with lauric acid, dicyclohexylcarbodiimide (DCC), 4-dimethylaminopyridine (DMAP) in Py/CH_2Cl_2. The acylation of (R)-4-hydroxy-1-methyl-2-pyrrolidinone (**4**) [18] led to (R)-4-acetoxy-1-methyl-2-pyrrolidinone (**5**), while the condensation with lauric acid afforded (R)-4-lauroyloxy-1-methyl-2-pyrrolidinone (**6**). (Scheme 1).

Starting from 3-amino-2-piperidinone [19], *N*-(2-oxopiperidin-3-yl)dodecanamide (**7**) was obtained by condensation with lauric acid which was then transformed by acylation into *N*-acetyl-*N*-(1-acetyl-2-oxopiperidin-3-yl) dodecanamide (**8**). The reaction of 3-amino-1-methyl-2-piperidinone (**9**) [20] with lauric acid, under the usual conditions, led to *N*-(1-methyl-2-oxopiperidin-3-yl)dodecanamide (**10**). (Scheme 2).

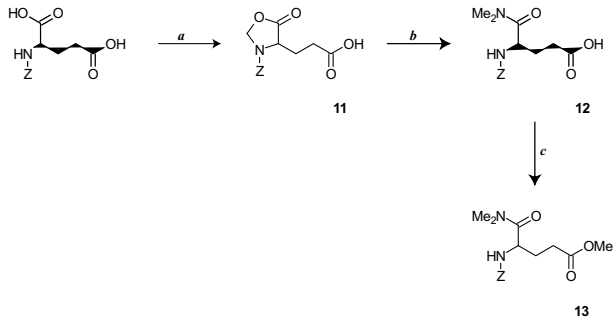
4-{[(Benzyloxy)carbonyl]amino}-5-(dimethylamino)-5-oxopentanoic acid (**12**) was prepared starting from N-[(benzyloxy)carbonyl]glutamic acid [21,22] through the cyclic intermediate **11**, and then converted into **13** by reaction with diazomethane. (Scheme 3).



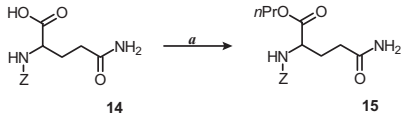
Scheme 1. (R)-4-Hydroxy-2-pyrrolidinone derivatives
a: Ac_2O, Py; ***b***: NaH, 4-chlorobenzyl chloride, DMF; ***c***: DCC, Py, lauric acid, DMAP, CH_2Cl_2; ***d***: TBSCl, 1*H*-imidazole, DMF; ***e***: NaH, MeI, DMF; ***f***: HCl, MeOH.



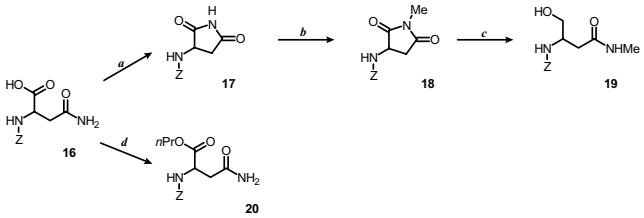
Scheme 2. 3-Amino-2-piperidinone derivatives
a: DDC, Py, lauric acid, DMAP, CH_2Cl_2; ***b***: Ac_2O, reflux; ***c***: Et_3N, $(Boc)_2O$, CH_2Cl_2; ***d***:NaH, MeI, DMF; ***e***: CF_3CO_2H.



Scheme 3. Glutamic acid derivatives
a: paraformaldehyde, *p*-toluenesulfonic acid, C_6H_6; ***b***: $Me_2NH{\cdot}HCl$, $NaOH/H_2O$; ***c***: CH_2N_2, $CHCl_3$.



Scheme 4. Glutamine derivatives
a: $SOCl_2$, *n*-PrOH.



Scheme 5. Asparagine derivatives
a: K_2CO_3, DMF; ***b***: *t*-BuOK, MeI, DMF; ***c***: $NaBH_4$, *i*-PrOH/toluene/H_2O; ***d***: $SOCl_2$, *n*-PrOH.

Starting from N^2-[(benzyloxy)carbonyl]glutamine (**14**), *n*-propyl N^2-[(benzyloxy)carbonyl]glutaminate (**15**) was synthesised by reaction with thionyl chloride and dry *n*-propanol. (Scheme 4).

3-[(Benzyloxy)carbonylamino]-4-hydroxy-N-methylbutanamide (**19**) was obtained starting from N^2-[(benzyloxy)carbonyl]asparagine (**16**), which was at first transformed into 3-[(benzyloxy)carbonylamino]pyrrolidine-2,5-dione (**17**) [23], then N-methylated [24] and successively reducted with $NaBH_4$ in *i*-propanol/toluene/water (6:1:1). Starting from **16**, *n*-propyl N^2-[(benzyloxy)carbonyl]-α-asparaginate (**20**) was obtained by reaction with thionyl chloride and dry *n*-propanol. (Scheme 5).

3. Results and Discussion

We here present data showing the anti-apoptotic activity of newly synthesised compounds.

The previously reported anti-apoptotic activity of carnitine was demonstrated in experimental systems where cell death was induced by triggering Fas. Therefore, experiments were performed to evaluate the influence that the synthesised

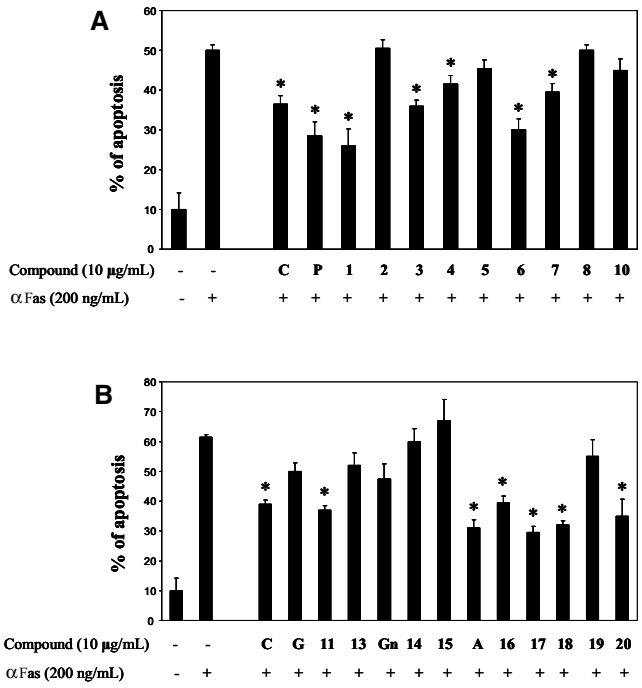


Fig. 1. Jurkatt cells (10^6 cell/mL) were incubated in the absence (-) or presence (+) of 200 ng/mL anti-Fas plus different compounds (10 μg/mL) as shown in the figure. The percentage of cells undergoing apoptosis was determined by PI staining. Representative data are shown with mean percentage apoptosis ± SD from three experiments. **C** = carnitine; **G** = glutamic acid; **Gn** = glutamine; **A** = asparagine. * P<0.05

compounds had on Fas-induced apoptosis. Human Jurkatt cell line was treated with anti-Fas mAb alone or in combination with different compounds and apoptosis was evaluated by propidium iodide (PI) staining and flow cytometry analysis as previously described [25].

Results of representative experiments are shown in Figures 1, 2 and 3. The results obtained when carnitine cyclic analogues and AA derivatives were tested at the concentration of 10 μg/mL are reported in Figure 1. The results from two different experiments (A and B), confirm previous observations that carnitine inhibits Fas-induced apoptosis. The results also indicate that the activities of some of the carnitine cyclic analogues (A) and some of the AA derivatives (B) are

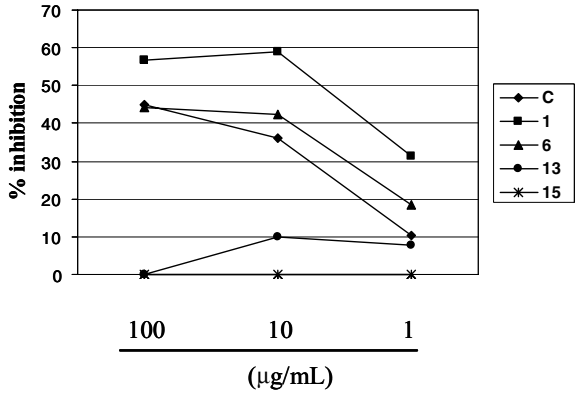


Fig. 2. Percentage of inhibition of anti-Fas-induced apoptosis in Jurkatt cells by different compounds in a dose-dependent manner. Percentage of cells undergoing apoptosis was determined by PI staining. **C** = carnitine.

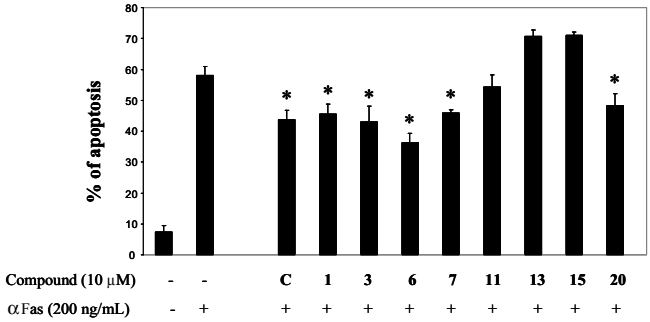


Fig. 3. Jurkatt cells (10^6 cell/mL) were incubated in the absence (-) or presence (+) of 200 ng/mL anti-Fas plus different compound (10 μM) as shown in the figure. The percentage of cells undergoing apoptosis was determined by PI staining. Representative data are shown with mean percentage apoptosis ± SD from three experiments. **C** = carnitine. * P<0.05

comparable to that of carnitine. In particular, some of the five-member (**P, 1, 3, 4, 6**) and one of the six-member (**7**) cyclic analogues, showed a significant inhibitory activity, while other five-member (**2, 5**) and six-member (**8, 10**) cyclic analogues did not significantly inhibit Fas-induced apoptosis (Figure 1-A). Results of the experiment, that tested the AA derivatives (Figure 1-B), indicate that the activities of some of the compounds (**A, 11, 16, 17, 18, 20**) were comparable to that of carnitine, while other compounds (**G, Gn, 13, 14, 15, 19**) did not significantly inhibit apoptosis.

The compounds were also tested at different concentrations ranging between 1 and 100 μg/mL. The results of a representative experiment, reported in Figure 2, confirm that **1** and **6** had an inhibitory activity comparable, or slightly superior, to that of carnitine, while **13** and **15** did not significantly inhibit apoptosis.

To further evaluate the inhibitory activity of some of the different compounds, experiments were performed using equimolar concentrations. The results reported in Figure 3 show that anti-Fas-induced apoptosis was inhibited when carnitine, carnitine cyclic analogues and AA derivatives were tested at the equimolar concentration of 10 μM. Carnitine was active at this concentration which was 5 times less than that used in the experiments reported in Figure 1, in which 10 μg/mL was equal to 50.5 μM. These results thus confirming that carnitine activity also occurred at lower concentrations (Figure 2). The results in Figure 3 indicate that the cyclic analogues **1, 3, 6, 7** and the AA derivative **20** showed anti-apoptotic activity, while **11, 13** and **15** did not inhibit Fas-induced apoptosis. In contrast to the results reported in Figure 1, **11** showed a slight but not significant inhibition and this was due to the lower concentration used with respect to that reported in Figure 1, in that 10 μM was equal to 2.9 μg/mL. As expected from the results reported in Figure 1, **13** and **15** were not active at the 10 μM concentration. All the other compounds listed in Figure 3 were significantly active as in the experiments reported in Figure 1. In comparison with the 10 μg/mL, 10 μM corresponds to a concentration reduction of 5 times for carnitine (10 μM is equal to 2 μg/mL), 7 times for **1** (10 μM is equal to 1.42 μg/mL), and 3 times for **3, 6** and **7** (10 μM is about 3.3 μg/mL), thus

indicating that these synthesised compounds (**1, 3, 6, 7**) have an activity comparable to that of carnitine.

Taking into consideration the small number of new compounds in every series, we could only advance a tentative hypothesis based on structure-activity relationships. In the series of (R)-4-hydroxy-2-pyrrolidinone derivatives we can note that by transforming the hydroxyl group into the corresponding lauroyl ester (compounds **3** and **6)** the activity was maintained or improved slightly depending on the simultaneous nitrogen alkylation. Applying this result to the 3-amino-2-piperidinone series, the transformation of the amino group into lauroyl amide maintained the anti-apoptotic activity (compound **7)** that decreased with the simultaneous alkylation of the endocyclic nitrogen (compounds **8** and **10**).

In the series of amino acids, N-benzyloxycarbonyl derivatives were synthesised to test the hypothesis that the Z-group plays a role in the modulation of apoptosis but it leads to a positive effect only in the case of five-member cyclic intermediates (compounds **11, 17** and **18**).

In conclusion, the preliminary results here described confirm that carnitine antagonises Fas-induced apoptosis and point out that some new carnitine analogues at least have similar activity. Future studies will be devoted to improve the activity by nitrogen quaternarization and to analyse the mechanism of anti-apoptotic activity.

4. Experimental Procedures

Melting points determined in capillary tubes (Electrothermal, Model 9100, melting point apparatus) were uncorrected. Element analysis was performed on a Carlo Erba element analyser 1106, and the data for C, H, and N are within ± 0.4% of the theoretical values. ^{1}H-NMR spectra were recorded at 200 MHz (Bruker DPX spectrometer) with Me_4Si as internal standard. Chemical shifts are given in ppm (δ) and the spectral data are consistent with the assigned structures. Column chromatography separations were carried out on Merck SiO_2 40 (mesh 70-230), SiO_2 60 (flash, mesh 230-400) or neutral Al_2O_3 activity III. Reagents and solvents were purchased from common commercial suppliers and used as received. Yields of purified product were not optimised. All starting materials were commercially available unless otherwise indicated.

4.1. (R)-4-Acetoxy-2-pyrrolidinone (1)

It was prepared starting from (R)-4-hydroxy-2-pyrrolidinone (**P**) according to the literature [17].

4.2. (R)-4-[(4-Chlorobenzyl)oxy]-2-pyrrolidinone (2)

NaH (60% mineral oil dispersion, 0.065 g, 1.63 mmol) was added portionwise to a solution of (0.15 g, 1.48 mmol) in dry DMF (5 mL) cooled to 0°C, and stirred for 15 min. 4-Chlorobenzyl chloride (0.28 g, 1.78 mmol) in dry DMF (5 mL) was then added dropwise and the mixture was maintained at room temperature overnight. The solution was poured into water, extracted with EtOAc, evaporated to dryness and the residue was purified on Al_2O_3 eluting with $CHCl_3$ to give **2** as an amorphous solid (0.10 g, 30%). ^{1}H-NMR ($CDCl_3$) δ: 2.45 (1H, dd, J = 18.0 and 2.0 Hz, CHC*H*HCO), 2.75 (1H, dd, J = 18.0 and 5.1 Hz, CHCH-*H*CO), 3.20 (1H, dd, J = 10.2 and 2.0 Hz, CHC*H*HNH), 3.50 (1H, dd, J = 10.2 and 5.1 Hz, CHCH*H*NH), 4.30-4.55 (3H, m, $CHOCH_2$), 7.10-7.35 (5H, m, aromatic H and NH).

4.3. (R)-4-Lauroyloxy-2-pyrrolidinone (3)

DCC (0.41 g, 1.98 mmol) and dry Py (0.22 mL) were added to a solution of lauric acid (0.40 g, 1.98 mmol) in dry CH_2Cl_2 (10 mL). A suspension of **P** (0.20 g, 1.98 mmol) and DMAP (0.24 g, 1.98 mmol) in dry CH_2Cl_2 (10 mL) was then added to the resulting mixture and maintained at room temperature for 48 h. After filtration under vacuum, the resulting solution was evaporated to dryness and chromatographed on flash SiO_2 eluting with cyclohexane/EtOAc 3:7. **3** was obtained as a white solid which crystallized from MeOH, 0.34 g, 60%, mp 89-90°C. ^{1}H-NMR ($CDCl_3$) δ: 0.90 (3H, t, J = 6.6 Hz, CH_3), 1.25-1.50 (16 H, m, aliphatic H), 1.55-1.70 (2H, m, $OCOCH_2CH_2$), 2.35 (2H, t, J = 7.6 Hz, $OCOCH_2CH_2$), 2.40 (1H, dd, J = 17.0 and 2.4 Hz, CHC*H*-HCO), 2.75 (1H, dd, J = 17.0 and 7.0 Hz, CHCH*H*CO), 3.40 (1H, dd, J = 11.5 and 1.5 Hz, CHC*H*HNH), 3.80 (1H, dd, J = 11.5 and 5.9 Hz, CHCH*H*NH), 5.40-5.50 (1H, m, CH_2CH CH_2), 6.80 (1H, bs, NH).

4.4. (R)-4-Hydroxy-1-methyl-2-pyrrolidinone (4)

It was prepared starting from **P** according to the literature [18].

4.5. (R)-4-Acetoxy-1-methyl-2-pyrrolidinone (5)

A mixture of **4** (0.17 g, 1.47 mmol), Ac_2O (0.15 g, 10.30 mmol) and dry Py (2.5 mL) was stirred at room temperature for 6 h, then poured into water and extracted with $CHCl_3$. The combined organic layers were washed with a saturated solution of NaCl, then with water and evaporated to dryness. The residue was chromatographed on flash SiO_2 eluting with $CHCl_3$ to give **5** as a yellow oil (0.13 g, 56.5%). ^{1}H-NMR (CD_3OD) δ: 2.10 (3H, s, $COCH_3$), 2.40 (1H, dd, J = 18.0 and 1.5 Hz, CHC*H*HCO), 2.75-2.90 (4H, m, NCH_3 and CHCH*H*CO), 3.45 (1H, dd, J = 11.5 and 1.6 Hz, CHC*H*HNH), 3.85 (1H, dd, J = 11.5 and 5.5 Hz, CHCH*H*NH), 5.25-5.35 (1H, m, CH_2CH CH_2).

4.6. (R)-4-Lauroyloxy-1-methyl-2-pyrrolidinone (6)

DCC (0.36 g, 1.74 mmol) and dry Py (0.2 mL) were added to a solution of lauric acid (0.35 g, 1.74 mmol) in dry CH_2Cl_2

(5 mL). A suspension of **4** (0.20 g, 1.74 mmol) and DMAP (0.21 g, 1.74 mmol) in dry CH_2Cl_2 (5 mL) was then added to the resulting mixture and maintained at room temperature for 48 h. After filtration under vacuum, the resulting solution was evaporated to dryness and chromatographed on flash SiO_2 eluting with $CHCl_3$/MeOH 99:1. **6** (0.42 g, 81%) was obtained as a white solid which crystallized from MeOH, mp 219-220°C. ^{1}H-NMR ($CDCl_3$) δ: 0.90 (3H, t, J = 6.7 Hz, CH_3), 1.25-1.47 (16H, m, aliphatic H), 1.60-1.75 (2H, m, $OCOCH_2CH_2$), 2.30 (2H, t, J = 7.6 Hz, $OCOCH_2CH_2$), 2.50 (1H, dd, J = 17.5 and 1.5 Hz, CHCHHCO), 2.85 (1H, dd, J = 17.5 and 7.0 Hz, CHCHHCO), 2.90 (3H, s, NCH_3), 3.35 (1H, dd, J = 11.5 and 1.5 Hz, CHCHHNH), 3.80 (1H, dd, J = 11.5 and 5.9 Hz, CHCHHNH), 5.25-5.35 (1H, m, CH_2CH CH_2).

4.7. N-*(2-Oxopiperidin-3-yl)dodecanamide (7)*

DCC (0.54 g, 2.63 mmol) and dry Py (0.30 mL) were added to a solution of lauric acid (0.53 g, 2.63 mmol) in dry CH_2Cl_2 (15 mL). A suspension of 3-amino-2-piperidinone [19] (0.30 g, 2.63 mmol) and DMAP (0.32 g, 2.63 mmol) in dry CH_2Cl_2 (10 mL) was then added to the resulting mixture and maintained at room temperature for 48 h. After filtration under vacuum, the resulting solution was evaporated to dryness and chromatographed on SiO_2 eluting with $CHCl_3$/MeOH 99:1. **7** (0.55 g, 70%) was obtained as a white solid which crystallized from MeOH, mp 154-156°C. ^{1}H-NMR ($CDCl_3$) δ: 0.92 (3H, t, J = 6.0 Hz, CH_3), 1.05-2.05 (21 H, m, aliphatic H), 2.25 (2H, t, J = 8.0 Hz, $COCH_2$), 2.60-2.70 (1H, m, CHHCH), 3.35-3.40 (2H, m, $NHCH_2$), 4.20-4.40 (1H, m, $CHCH_2$), 6.00 (1H, bs, piperidinonic NHCO), 6.45 (1H, bs, CONH).

4.8. N-*Acetyl*-N-*(1-acetyl-2-oxopiperidin-3-yl) dodecanamide (8)*

A solution of **7** (0.15 g, 0.51 mmol) in Ac_2O (5 mL) was refluxed for 3 h. The solution was poured into water and extracted with EtOAc. The combined organic layers were evaporated to dryness and purified on flash SiO_2 eluting with $CHCl_3$/MeOH 9:1 to furnish **8** as a colourless oil (0.04 g, 20%). ^{1}H-NMR ($CDCl_3$) δ: 0.90 (3H, t, J = 6.8 Hz, CH_3), 1.05-2.05 (21H, m, aliphatic H), 2.20 (2H, t, J = 7.8 Hz, $COCH_2$), 2.60-2.70 (1H, m, CHHCH), 2.95 (3H, s, NCH_3), 3.20-3.40 (2H, m, CH_2N), 4.15-4.30 (1H, m, $CHCH_2$), 6.55 (1H, bs, CONH).

4.9. 3-Amino-1-methyl-2-piperidinone (9)

It was prepared according to the literature with MeI instead of iodomethyl phenylacetate [20].

4.10. N-*(1-Methyl-2-oxopiperidin-3-yl)dodecanamide (10)*

DCC (0.40 g, 1.95 mmol) and dry Py (0.20 mL) were added to a solution of lauric acid (0.39 g, 1.95 mmol) in dry CH_2Cl_2 (15 mL). A suspension of 3-amino-1-methyl-2-piperidinone **9** (0.25 g, 1.95 mmol) and DMAP (0.24 g, 1.95 mmol) in dry CH_2Cl_2 (5 mL) was then added to the resulting mixture and maintained at room temperature for 48 h. After filtration under vacuum the resulting solution was evaporated to dryness and chromatographed on SiO_2 eluting with $CHCl_3$/MeOH 99:1. **10** (0.42 g, 70%) was obtained as a white solid which crystallized from cyclohexane/EtOAc, mp 71-72°C. ^{1}H-NMR ($CDCl_3$) δ: 0.90 (3H, t, J = 6.8 Hz, CH_3), 1.05-2.05 (21H, m, aliphatic H), 2.20 (2H, t, J = 7.8 Hz, $COCH_2$), 2.60-2.70 (1H, m, CHHCH), 2.95 (3H, s, NCH_3), 3.20-3.40 (2H, m, CH_2N), 4.15-4.30 (1H, m, $CHCH_2$), 6.55 (1H, bs, CONH).

4.11. 3-{3-[(Benzyloxy)carbonyl]-5-oxo-1,3-oxazolidin-4-yl}propanoic acid (11)

It was prepared starting from N-[(benzyloxy)carbonyl]-glutamic acid according to the literature [21].

4.12. 4-{[(Benzyloxy)carbonyl]amino}-5-(dimethylamino)-5-oxopentanoic acid (12)

It was prepared starting from **11** according to the literature [22].

4.13. Methyl 4-{[(benzyloxy)carbonyl]amino}-5-(dimethylamino)-5-oxopentanoate (13)

Diazomethane 0.15M solution in Et_2O was added dropwise to a solution of **12** (0.15 g, 0.49 mmol) until the solution turned yellow. The mixture was evaporated to dryness and the residue was chromatographed on flash SiO_2 eluting with cyclohexane/EtOAc 1:1 to give **13** (0.12 g, 76%) as an oil. ^{1}H-NMR ($CDCl_3$) δ: 1.70-1.90 and 2.05-2.25 (each 1H, m, $CHCH_2CH_2$), 2.40-2.60 (2H, m, $CHCH_2CH_2$), 3.00 and 3.20 (each 3H, s, $N(CH_3)_2$), 3.75 (3H, s, OCH_3), 4.75-4.85 (1H, m, $CHCH_2CH_2$), 5.15 (2H, s, CH_2Ph), 5.80 (1H, d, J = 8.1 Hz, NH), 7.30-7.40 (5H, m, aromatic H).

4.14. n-*Propyl* N^2-*[(benzyloxy)carbonyl]glutaminate (15)*

Thionyl chloride (0.25 g, 0.21 mmol) was added dropwise to a cooled suspension of N^2-[(benzyloxy)carbonyl] glutamine **14** (0.20 g, 0.71 mmol) in dry *n*-propanol (5 mL). The mixture was allowed to warm to room temperature and stirred for 4 h. After evaporation to dryness, the residue was purified by chromatography on SiO_2 eluting with $CHCl_3$/MeOH 9:1 to give **15** (0.15 g, 65%), mp 108-113°C. ^{1}H-NMR (DMSO-d_6) δ: 0.95 (3H, t, J = 7.5 Hz, CH_3), 1.50-1.70 (2H, m, CH_2CH_3), 1.72-1.90 and 1.92-2.15 (each 1H, m, $CHCH_2CH_2$), 2.17-2.25 (2H, m, $CHCH_2CH_2$), 3.95-4.15 (3H, m, $CHCH_2CH_2$ and OCH_2CH_2), 5.10 (2H, s, CH_2Ph), 6.85 and 7.30 (each 1H, bs, $CONH_2$), 7.32-7.45 (5H, m, aromatic H), 7.78 (1H, d, J = 6.1 Hz, NH).

4.15. 3-[(Benzyloxy)carbonylamino]pyrrolidine-2,5-dione (17) [23]

K_2CO_3 (2.85 g, 20.65 mmol) was added to a cooled solution of N^2-[(benzyloxy)carbonyl]asparagine **16** (0.50 g, 1.87 mmol) in dry DMF (10 mL). The mixture was allowed to warm to room temperature and stirred for 5 h, the solution was then poured into water, neutralized with 3N HCl and extracted with EtOAc. The combined organic layers were evaporated to dryness and the residue was flash chromatographed on SiO_2 eluting with $CHCl_3$/MeOH 94:6 to give **17** (0.36 g, 77%) as an amorphous solid. ^{1}H-NMR ($CDCl_3$) δ: 2.75 (1H, dd, J = 18.0 and 5.7 Hz, COC*H*H), 3.05 (1H, dd, J = 18.0 and 8.8 Hz, COCH*H*), 4.35-4.45 (1H, m, C*H*NH), 5.10 (2H, s, CH_2Ph), 6.00 (1H, d, J = 6.8 Hz, CHN*H*), 7.30-7.40 (5H, m, aromatic H), 9.35 (1H, bs, NH).

4.16. 1-Methyl-3-[(benzyloxy)carbonylamino]pyrrolidine-2,5-dione (18) [24]

t-BuOK (0.11 g, 0.97 mmol) was added in one portion to a solution of **17** (0.20 g, 0.81 mmol) in dry DMF (2 mL). The resulting mixture was stirred for 15 min followed by the dropwise addition of MeI (0.13 g, 0.97 mmol) in dry DMF (1 mL). The solution was stirred for an additional 2 h and then poured into water and extracted with EtOAc. The combined organic layers were evaporated to dryness and the residue was chromatographed on SiO_2 eluting with $CHCl_3$/MeOH 99:1. **18** (0.17 g, 80%) was obtained as a white solid which crystallized from MeOH, mp 104-106°C. ^{1}H-NMR ($CDCl_3$) δ: 2.80 (1H, dd, J = 17.6 and 5.2 Hz, COC*H*H), 2.85-3.10 (4H, m, NCH_3 and COCH*H*), 4.25-4.40 (1H, m, C*H*NH), 5.15 (2H, s, CH_2Ph), 5.75 (1H, d, J = 5.8 Hz, CHN*H*), 7.25-7.50 (5H, m, aromatic H).

4.17. 3-[(Benzyloxy)carbonylamino]-4-hydroxy-N-methylbutanamide (19)

$NaBH_4$ (0.22 g, 5.72 mmol) was added portionwise to a cooled solution of **18** (0.50 g, 1.91 mmol) in *i*-propanol/toluene/water (6:1:1). The mixture was stirred at room temperature for 3 h, then evaporated to dryness. The residue was purified by flash chromatography on SiO_2 eluting with CH_2Cl_2/MeOH 9:1 to give **19** (0.10 g, 20%) as a white solid which crystallized from MeOH, mp 164-166°C. ^{1}H-NMR (DMSO-d_6) δ: 2.10-2.45 (2H, m, CH_2CHNH), 2.55 (3H, d, J = 4.5 Hz, NHCH_3), 3.20-3.45 (2H, m, CH_2OH), 3.75-3.90 (1H, m, CH_2C*H*NH), 4.75 (1H, t, J = 5.5 Hz, OH), 5.00 (2H, s, CH_2Ph), 7.05 (1H, d, J = 8.3 Hz, CON*H*CH), 7.25-7.45 (5H, m, aromatic H), 7.70-7.80 (1H, m, N*H*CH_3).

4.18. n-*Propyl* N^2*-[(benzyloxy)carbonyl]-asparaginate (20)*

Thionyl chloride (0.66 g, 5.64 mmol) was added dropwise to a cooled suspension of **16** (0.50 g, 1.88 mmol) in dry *n*-propanol (12 mL). The mixture was allowed to warm to room temperature and stirred for 4 h. After evaporation to dryness, the residue was purified by chromatography on SiO_2 eluting with $CHCl_3$/MeOH 8:2 to give **20** as a white solid (0.43 g, 75%), mp 122-125°C. ^{1}H-NMR (DMSO-d_6) δ: 0.88 (3H, t, J = 7.3 Hz, CH_3), 1.45-1.70 (2H, m, C$H_2$$CH_3$), 2.35-2.70 (2H, m, CHC$H_2$), 4.00 (2H, t, J = 6.3 Hz, OC$H_2$$CH_2$), 4.35-4.55 (1H, m, C*H*CH_2), 5.05 (2H, s, CH_2Ph), 7.00 (1H, bs,), 7.20-7.55 (6H, m, aromatic H and CONH*H*), 7.65 (1H, d, J = 8.1 Hz, NH).

4.19. Cell culture

The human Jurkatt T cell line was maintained in RPMI 1640 medium (HyClone, Logan, UT) supplemented with 10% FBS (HyClone), 2 mM L-glutamine (Life Technologies), and 10 μg/mL gentamicin (Life Technologies). Cells were grown at 37°C in a humidified atmosphere consisting of 5% CO_2. For the experiments, cells in log phase growth were resuspended at a density of $1x10^6$ cells/mL and incubated in the absence or presence of 200 ng/mL mouse anti-human Fas mAb (Oncogene, San Diego, CA) for 30 min and subsequently incubated with L-carnitine and its analogues.

All compounds were dissolved in DMSO and diluted at the concentration indicated in the figures with RPMI 1640 medium supplemented with 10% FBS, 2mM L-glutamine, and 10 μg/mL gentamicin.

In all experiments vehicle alone (DMSO) at same concentration of working solution has been used as control.

4.20. Apoptosis evaluation by PI solution

Apoptosis was measured by flow cytometry as described elsewhere [25]. After culturing, cells were centrifuged and the pellets were gently resuspended in 1.5 mL hypotonic PI solution (PI, 50 μg/mL in 0.1% sodium citrate plus 0.1% Triton X-100, Sigma). Tubes were kept at 4°C in the dark overnight. The PI-fluorescence of individual nuclei was measured by flow cytometry with standard FACScan equipment (Becton Dickinson). The nuclei traversed the light beam of a 488 nm Argon laser. A 560 nm dichroid mirror (DM 570) and a 600 nm band pass filter (band width 35 nm) were used to collect the red fluorescence due to PI DNA staining, and data were recorded in logarithmic scale in a Hewlett Packard (HP 9000, model 310) computer. The percentage of apoptotic cell nuclei (sub-diploid DNA peak in the DNA fluorescence histogram) was calculated with specific FACScan research software (Lysis II).

Acknowledgements

This study was supported by Sigma Tau – Industrie Farmaceutiche Riunite S.p.A. and Mendes S.r.l. (contracts: 01/ALSI/2001 and ALSI/2002/C.R./n.3).

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