Oleic Acid Amides With Potential Pharmacological Effects in the Overweight Treatment

CATALINA NEGUT[1]*, LUCIA PINTILIE[1], CONSTANTIN TANASE[1], DENISA IOANA UDEANU[2]*, CONSTANTIN DRAGHICI[3], CRISTIAN MUNTEANU[4], ELENA MOROSAN[2]

[1]National Institute for Chemical Pharmaceutical Research and Development, 112 Vitan Av., 031299, Bucharest, Romania
[2]University of Medicine and Pharmacy Carol Davila, Bucharest, Faculty of Pharmacy, Department of Clinical Laboratory and Food Safety, 6 Traian Vuia Str., 020956, Bucharest, Romania
[3]Organic Chemistry Center C.D.Nenitescu, 202B Splaiul Independentei, 060023, Bucharest, Romania
[4]Romanian Academy-Institute of Biochemistry (IBAR), 296 Splaiul Independentei, 060031, Bucharest, Romania

Fatty acid amide analogues were synthesized from oleic acid, activated by 1,1'-carbonildiimidazole to oleoylimidazole and amines, and characterized by IR, MS, ^{1}H- and ^{13}C-NMR spectra. The compounds were investigated for their influence on body weight and food-intake effects on an obesity-induced mouse model.

Keywords: *Fatty acid amide analogues, ^{1}H- and ^{13}C-NMR, IR, MS spectra, food-intake, body weight regulation.*

Obesity and overweight are considered two of the most important medical problems of current day, due to the effect on general health of population and the diseases associated or further developing like metabolic syndrome, cardiovascular disease, type II diabetes mellitus (T2D) [1] and some forms of cancer. Obesity is a condition that can be very well prevented, and compared to applying treatment, prevention strategies are more effective and not expensive [2, 3].

For some years we are interested to find new candidates for treatment of obesity and reduced food-intake and studied the possibility to find this application in the field of fatty acid amides. It is known that fatty acid amides are not only important for industrial applications [4] and even as ingredient of foods. Due to increased knowledge of their important mediator effect as endogenous molecules for many biological activities in different tissue types, the biochemical, pharmacological [5] studies are extended to new analogues of fatty acid amides. For ex., researches about these compounds have been shown to be involved in many organ systems, such immune, energy balance, food intake, metabolic homeostasis, cardiovascular, depressive effects, fertility, pain, and neuroprotection [6-8]. The fatty acid amides may also have a role in cancer biology because of its effects on cell proliferation [9, 10]. This wide implication of fatty acid amides is closely associated with the presence of fatty acids in current food and due to the following metabolism of these compounds. For these studies, a lot of new fatty acid amides were synthesized and these were obtained by traditional methods for obtaining amides [11].

We already synthetized a library of fatty acids amide analogues and used for the analysis of specific biomarkers from biological fluids and performed the preliminary pharmacological activities regarding in the decreasing body weight and food intake after daily administration on Wistar rats [12] and intraperitoneal administration on mice [13]. A number of our compounds have a similar profile with that of oleoylethanolamide (OEA), considered an endogenous compound involved in positive effect for reducing food-intake and so to reduce obesity. The most significant and promising effect for reducing food-intake and reducing the body weight, greater than that obtained with OEA, was obtained by including in the daily food of mice and Wistar rats the optically active oleamide, (Z)-N-[(1S)-2-hydroxy-1-(phenylmethyl)ethyl]-9-octadecenamide (PhEO). The preliminary results are very optimistic for developing a new drug for obesity treatment from this structural class of compounds.

The importance of fatty acid amide analogues determined us to increase the library of fatty acid amides, exploring new amines to be used for obtaining new analogues in hope to find even more favorable activity than that of PhEO.

Experimental part

Melting point was determined on OptiMelt. TLC was performed on Merck silicagel 60 or 60F_{254} plates and spots were developed under UV light, with iodine and/or 15% H_2SO_4 in methanol and heating at 120-140°C. The ^{1}H- and ^{13}C-NMR spectra were recorded on a Varian Gemini 300 BB instrument, operating at 300 MHz for ^{1}H-NMR and at 75 MHz for ^{13}C-NMR, using $CDCl_3$ as solvent and TMS as internal standard. The IR spectra (ATR) were recorded on a Vertex 70 Brucker instrument. Mass spectra were recorded at 100000 resolution (m/z 400), in positive mode on an LTQ Orbitrap Velos Pro, by injecting a solution of 100 pmol/μL in 0.1 % formic acid in methanol. All fragmentations were performed using CID (Collision Induced Dissociation) and the fragments were recorded in the linear ion trap.

Biochemical analyses

The plasmatic parameters (total cholesterol, LDL-cholesterol, triglycerides and glycaemia) were determined using a Cormay-multi biochemical analyser with specific reagents.

Statistical analyses of data

Statistical significance of the experimental data was performed by ANOVA and t-student tests using Graph Pad Prism soft. The results were considered statistical significant in case of $p<0.05$ and not significant (NS) in case of $p>0.05$.

Chemistry

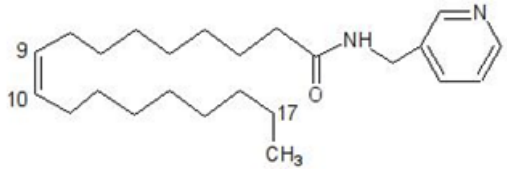
General procedure for synthesis of amides N1-N3

Oleic acid amide analogues **N1-N3**, were synthesized from oleic acid (12 mmoles) by reaction with 1,1'-

* *Phone: (+40)724205937; (+40)730567585*

carbonildiimidazole (CDI) (12.8 mmoles) in dichloromethane (100 mL) for 2 h and then the activated oleoylimidazole solution added dropwise to a stirred solution of 20 mmoles amine and 1.11 mmoles 4-dimethyl-aminopyridine in CH_2Cl_2 (50 mL); the reaction mixture was stirred for 48 h at r.t., monitoring the reaction by TLC. This procedure was previously successfully applied for synthesis of another eight compounds [12, 13] by a published procedure for obtaining oleamide analogues presented in the macamides type [14].

- Synthesis of N-(pyridin-3-yl methyl)oleamide, **N1**



TLC (Silicagel, eluent: ethyl acetate-methanol, 90:13, $R_{f\,N1}$ = 0.71). The crude product was dissolved in hexane at reflux, than cooled at room temperature, resulting 4.12g (92.7%) of pure product **N1** as waxy.

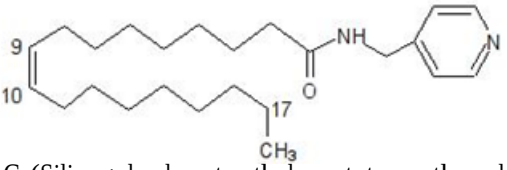
IR: 3294ms (νNH), 3007w ($\nu_{=C-H}$), 2957m, 2921vs (νCH_{2asim}), 2851s (νCH_{2sim}), 1635s (νC=ONHR sec. amide), 1541s (νC=O sec. amide, band II), 1463m, 1426m; 1224w, 1031w, 800w, 714m,

^{1}H-NMR ($CDCl_3$, δ ppm, *J* Hz): 8.46 (brs, 1H, H-2', H-6'), 7.62 (dt, 1H, H-4', 1.9, 8.0), 7.26 (dd, 1H, H-5', 4.8, 8.0), 6.43 (br s, 1H, NH), 5.38 (m, 2H, H-9, H-10), 4.41 (d, 2H, H-CH_2N, 5.8), 2.21 (t, 2H, H-2, 7.4), 2.10-1.90 (m, 4H, 2H-8, 2H-11), 1.70-1.58 (m, 2H, H-3), 1.39-1.20 (m, 20H), 0.88 (t, 3H, H-18, 6.3),

^{13}C-NMR ($CDCl_3$, δ ppm): 171.45 (C-1), 149.07 (C-2'), 148.71 (C-6'), 135.67 (CH-4'), 134.43 (C-3'), 130.05, 129.74 (2C, C-9, C-10), 123.63 (C-5'), 40.89 (CH_2-NH), 36.62 (C-2), 31.94, 29.80, 29.75, 29.56, 29.36, 29.18, 29.03, 27.26, 27.21, 25.79 (12CH_2), 22.73 (C-17), 14.17 (C-18),

HR-MS, calc. for $C_{24}H_{40}N_2O$, $[M+H]^+$: 373.32134, found: 373.32026; fragments: 109 ($C_6H_9N_2$), 135 ($C_7H_7N_2O$), 355 ($C_{24}H_{39}N_2$) and minor fragments of consecutive olefinic backbone fragmentation.

-Synthesis of N-(pyridin-4-yl methyl)oleamide, **N2**



TLC (Silicagel, eluent: ethyl acetate-methanol, 90:13, $R_{f\,N2}$ = 0.44). The crude product was purified by pressure chromatography (eluent: ethyl acetate-methanol, 90:13), resulting 3.04 g (68%) of pure product **N2** as oil.

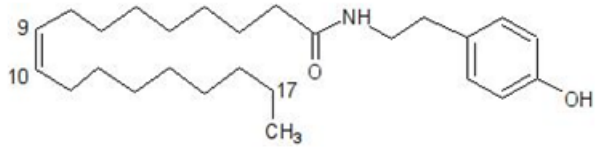
IR: 3285br m (νNH), 3005w ($\nu_{=C-H}$), 2923vs (νCH_{2asim}), 2853s (νCH_{2sim}), 1650s (νC=ONHR sec. amide), 1603m, 1543br m (νC=O sec. amide, band II), 1464m, 1416m, 1262w, 793w, 730w,

^{1}H-NMR ($CDCl_3$, δppm, *J* Hz): 8.43 (d, 2H, H-2', 5.5), 7.10 (d, 2H, H-3', 5.5), 6.38 (br s, 1H, NH), 5.32 (m, 2H, H-9, H-10), 4.36 (d, 2H, H-CH_2N, 6.0), 2.18 (t, 2H, H-2, 7.4), 2.00-1.84 (m, 4H, 2H-8, 2H-11), 1.65-1.55 (m, 2H, H-3), 1.37-1.15 (m, 20H), 0.81 (t, 3H, H-18, 6.6),

^{13}C-NMR ($CDCl_3$, δ ppm): 173.59 (C-1), 149.81 (C-2'), 148.06 (C-4'), 130.14, 129.78 (2C, C-9, C-10), 122.43 (2C-3'), 42.34 (CH_2-NH), 36.67 (C-2), 31.99, 29.85, 29.79, 29.60, 29.39, 29.34, 29.22, 27.32, 27.26, 25.82 (12CH_2), 22.76 (C-17), 14.19 (C-18),

HR-MS, calc. for $C_{24}H_{40}N_2O$, $[M+H]^+$: 373.32134, found: 373.32072; fragments: 109 ($C_6H_9N_2$), 135 ($C_7H_7N_2O$), 355 ($C_{24}H_{39}N_2$) and minor fragments of consecutive olefinic backbone fragmentation.

-Synthesis of (Z)-N-[2-(4-hydroxyphenyl)ethyl]octadec -9-enamide, **N3**



TLC (Silicagel, eluent: ethyl-acetate-hexane-acetic acid, 5:4:0.1, $R_{f\,N3}$ = 0.56). The crude product was crystalized from hexane, resulting 0.55g (11.4%) pure product **N3**, m.p. 62.9-63.7°C; the filtrate was concentrated and purified by pressure chromatography, resulting 0.75g of pure compound **N3**,

IR: 3415br m (νOH), 3298br m (νNH), 3005w ($\nu_{=C-H}$), 2956m, 2920vs (νCH_{2asim}), 2872m, 2849s (νCH_{2sim}), 1638s νC=ONHR sec. amide), 1614m, 1555br ms (νC=O sec. amide, band II), 1513m, 1365w, 1252m, 1174w, 824w,

^{1}H-NMR ($CDCl_3$, δ ppm, *J* Hz): 7.00 (d, 2H, H-3', 8.2), 6.81 (d, 2H, H-2', 8.2), 6.63 (br s, 1H, NH), 5.36 (m, 2H, H-9, H-10), 3.48 (q, 2H, CH_2N, 6.9), 2.72 (t, 2H, H-5', 6.9), 2.13 (t, 2H, H-2, 7.7), 2.09-1.85 (m, 4H, 2H-8, 2H-11), 1.65-1.55 (m, 2H, H-3), 1.40-1.10 (m, 20H), 0.88 (t, 3H, H-18, 6.6),

^{13}C-NMR ($CDCl_3$, δ ppm): 173.95 (C-1), 155.30 (C*q*, C-1'), 149.80 (C-2'), 129.94 (C-4'), 130.11, 129.85 (2C, C-9, C-10), 129.80 (2C-3'), 115.75 (2C-2'), 40.99 (CH_2-NH), 36.94 (C-2), 34.85 (CH_2-Ph), 32.00, 29.87, 29.81, 29.62, 29.42, 29.33, 29.23, 27.33, 27.27, 25.85 (12CH_2), 22.78 (C-17), 14.22 (C-18),

HR-MS, calc. for $C_{26}H_{43}NO_2$, $[M+H]^+$: 402.336656, found: 402.33598; fragments: 121 (C_8H_9O), 138 ($C_8H_{12}NO$) and minor fragments of consecutive olefinic backbone fragmentation.

Materials and methods

Experimental obesity-induced model in mice

Albino swiss (NMRI) male mice weighing 18±2g were purchased from the Animal Biobase of the University of Medicine and Pharmacy Carol Davila, Bucharest. Animals were kept in standard laboratory conditions and were fed twice a day and received water *ad libitum.* The experiment was performed in compliance with European Communities Council Directive 2010/63 and Ordinance No. 37 of the Romanian Government from 2nd February, 2002.

For 3 weeks before the experiment, animals received a hypercaloric diet according to other obesity-induced model described by the scientific literature [15]. The obese animals were selected for the study and distributed in 4 groups of 5 animals each: three groups treated with fatty acid amide analogues and an obese control group. A control group of 5 normal weight animals was used for the study and was fed with standard food.

The animals were intraperitoneal (i.p.) treated daily for 10 days as it follows:

Group 1-5.3 mg/Kg bw **N1** (N-(pyridin-3-yl methyl) oleamide);

Group 2-5.3 mg/Kg bw **N2** (N-(pyridin-4-yl methyl) oleamide);

Group 3-5.7 mg/ Kg bw **N3** ((Z)-N-[2-(4-hydroxyphenyl) ethyl]octadec-9-enamide);

Group 4-obese control group - 10mL/Kg bw physiological serum;

Group 5-normal weight control group - 10mL/Kg bw physiological serum.

The i.p. doses used for the treatment are molar equivalent with the therapeutic dose recommended for intraperitoneal administration of oleoylethanolamide [5mg (15μM)/kg bw] described by scientific literature [16, 17].

The hypercaloric diet was maintained for the 10 days of the treatment in obesity-induced groups (1-4), while the normal weight control group received standard food.

The animal body weights were measured in days 1, 3, 5, 7 and 10 of the treatment. The food consumption was determined 3 hours after the administration.

On the last day of the treatment, after two hours from the last administration, the animals were anesthetized with ethyl ether and the blood was collected for plasmatic lipid profile and glycemia.

Results and discussions

Chemistry

Synthesis of oleic acid amide analogues

For a period of time we are interested in the synthesis of a library of oleamide analogues and used in a project for satiety control, by reducing the food-intake and as a consequence, but not only, to reduce body weight and so to prevent the appearance of diabetes and the associate diseases.

So we first synthesized N-[2-(4-methoxyphenyl) ethyl]oleamide [18] by amidation of methyl oleate with 4-methoxyphenethylamine catalyzed by sodium methoxide. By the same procedure we obtained PhEO as optically active compound and N-(2-phenylethyl)oleamide; oleoylethanolamide (OEA) was also obtained by us using the same procedure [12]. Other eight oleamide compounds, N-(1-naphthyl)oleamide, N-cyclohexyloleamide, N-adamantyloleamide, N-(4-methylpiperazin-1-yl)oleamide, N-(2-(dimethylamino) ethyl)oleamide, N-(3-(diethylamino)propyl)oleamide, N-(2-hydroxy-3-phenoxypropyl)-N-isopropyloleamide, N-[2-naphthyl] oleamide were obtained by a second procedure from oleic acid, activated by carbonyldiimidazole, and primary amines [13], a procedure used in the field for synthesis of makamide's oleamide compounds [14].

By this established procedure, now we present the synthesis of the following oleic acid amide analogues **N1-N3**, in which the primary amines are separated from the aromatic ring (pyridine or phenyl) by a methylene or ethylene spacer: 3-methylpyridinyl, 4-methylpyridinyl and 2-(4-hydroxyphenyl)ethyl (Scheme 1). In these compounds it is waited to be a different influence of the aromatic groups, 3- or 4-substituted pyridine, respectively 4-hydroxyphenyl fragment. The structure of the new amide moieties could link in a favorably positions to the corresponding receptors (this moieties have structural elements to favor the link to receptor, due to aromatic character, nitrogen of pyridine fragment, 4-hydroxyphenyl and a spencer element: CH_2, respectively CH_2CH_2) and could be find in a positive effect of their biological activity, related in this case to reduce the food-intake and consequently the body weight. The compound **N2**, obtained as oil, was purified by pressure chromatography (and also the mother liquid of compound **N3**) and the compounds **N1** and **N3** were directly crystallized from hexane, first as waxy and the second, crystallized.

Until now, some of fatty acid amide analogues, have been tested for the analysis of specific biomarkers from biological fluids. Six new stochastic microsensors based on physical immobilization of oleamides in graphite paste, were used for the assay of the model analyte carcinoembryonic antigen (CEA) in the human whole blood. The results obtained using the proposed method were in good agreement with those obtained using the standard method (ELISA) [19]. Another two stochastic sensors based on oleamides physically immobilized on graphite paste were designed and characterized for the fast screening of whole blood samples of Wistar rat for Leptin, IL-6 and PAI-1 [20]. The novelty is given by the utilization of another two oleamides for the modification of diamond paste with three biogenic amines: histamine, putrescine, and cadaverine, used as model analytes for fast screening of wines [21]. This application of the new oleamide type analogues will be presented separately.

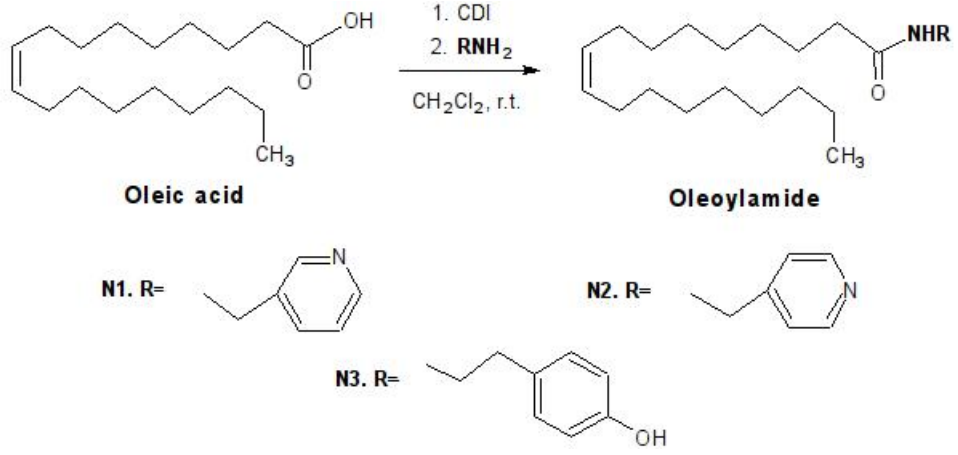
In the present, the fatty acid amide analogues were tested for their potential pharmacological use for decreasing food consumption and regulating the body weight using an animal model of induced obesity in mice.

^{1}H-, ^{13}C-NMR, IR and MS

^{1}H- and ^{13}C-NMR spectra presents the signals for the protons and carbon atoms characteristic for the amide moiety of the molecule together with the signals characteristic for the oleic acid moiety, confirming the structure of the molecule.

IR spectrum of the compounds presents an intense band at ~ 3300 for νNH, a very intense band at 1635-1650 cm^{-1} (CO) and a second intense band at 1541-1555 cm^{-1}, characteristics for an amide group. For CH_2 groups of the oleic moiety of the compounds there are present a very intense band at 2920-2923 cm^{-1} (νCH_{2asim}) and an intense band at 2849-2853 cm^{-1} (νCH_{2sim}).

HR-MS spectra gives molecular peaks corresponding to isotope peaks of compounds at $[M+H]^+$. Fragments for the amide moiety are readily visible for all compounds. In the same time the fragments for oleic moiety are ending with small fragments for consecutive olefinic skeleton



Scheme 1. Synthesis of compounds **N1-N3** from oleic acid and amines using CDI for activating carboxyl group

Table 1

THE INFLUENCE OF THE TREATMENT WITH FATTY ACID AMIDE ANALOGUES ON BODY WEIGHT IN OBESITY-INDUCED MICE

	Day 1 Mean±SD		Day 3 Mean±SD		Day 5 Mean±SD		Day 7 Mean±SD		Day 10 Mean±SD		t-Test Day 1 vs. Day 10
N1	38.8	±2.27	37.8	±2.35	38.0	±2.32	38.4	±2.00	38.0	±1.37	NS
N2	37.2	±0.34	37.6	±1.15	35.5	±1.30	34.6	±2.24	34.7	±0.99	P<0.05
N3	34.6	±0.99	33.8	±0.99	35.3	±1.19	36.6	±1.09	35.1	±1.57	NS
Obese	28.8	±4.45	30.2	±4.62	32.1	±4.33	33.9	±4.81	36.1	±4.94	P<0.001
Normal	20.5	±3.01	20.9	±2.88	21.2	±2.86	21.7	±2.84	22.5	±2.78	P<0.001
ANOVA	P<0.0001		P<0.0001		P<0.0001		P<0.0001		P<0.0001		

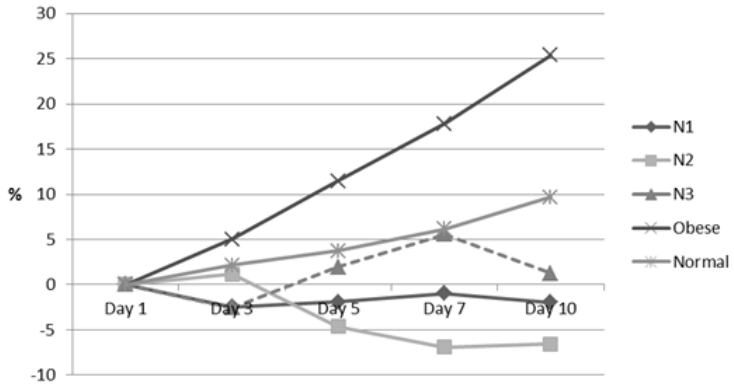


Fig. 1. The effect (%) of the treatment with fatty acid amide analogues on body weight in obesity-induced mice (**N1, N2, N3,** Obese - Obesity-induced control group, Normal - Normal weight control group)

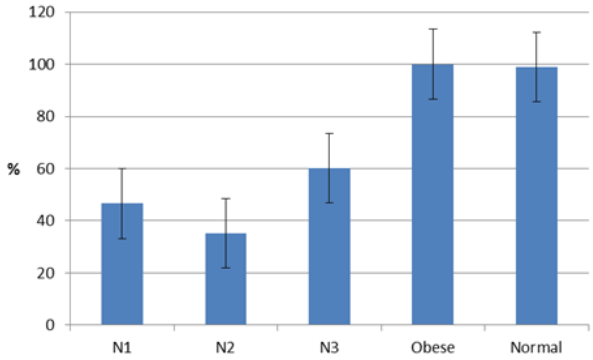


Fig. 2. The influence of the treatment with fatty acid amide analogues on food-intake in an obesity-induced mouse model. The efects are expressed in % comparing to the obese group (100%). (**N1, N2, N3,** Obese - Obesity-induced control group, Normal - Normal weight control group), bars in the graph represent standard errors

fragmentation, the corresponding ions being separated by 14 units (CH_2).

The analytical data fully confirmed the molecular structure of the fatty acid amide synthesized compounds.

Pharmacological tests

The influence of the treatment with fatty acid amide analogues on the body weight in an obesity-induced animal model

The effects on the body weight of the treatment with fatty acid amide analogues in obesity-induced mice are presented in table 1 and figure 1.

Statistical analyses were performed by one-way ANOVA within each day and t-student test to evaluate the differences between the body weight in day 1 and after 10 days of treatment. Results were considered as NS = not significant with $p>0.05$, significant for $p<0.05$ and high significant for $p<0.01$.

The hypercaloric diet induced an increase of 25% ($p<0.001$) of the body weight after 10 days in obese animal group comparing to a 10% increase of the body weight in the normal weight control group. The increasing of the weights was constant during the study in both control groups.

The i.p. treatment with fatty acid amide analogues decreased the body weight comparing with the obese control group after 10 days of administration. The obese animals from groups treated with **N1** and **N3** presented no significant variations ($p>0.05$) of the body weight during the period of the administration. Comparing to day 1, the animals treated with **N3** presented fluctuations on body weight during the study with a slightly increase by 5.8% after 7 days ($p<0.001$) followed by a decrease on the day 10 much closed to the initial body weight ($p>0.05$). The **N2** analogue treatment decreased the body weight by 7% ($p<0.05$) after 10 days of i.p. administration.

The influence of the treatment with fatty acid amide analogues on food-intake in an obesity-induced mice model

The treatment with the compounds significantly decreased the food consumption by 53, 65 and 40% after 3 h from the administration of **N1**, **N2** and respectively **N3** comparing to the obese group. The normal weight group received a standard diet and the quantity of food consumption was similar to the obese group which received a hypercaloric diet.

The decrease in food-intake may be the reason for the regulating the body weight in case of the obese animals treated with the new compounds. A significant pharmacological effect was obtained in case of **N2** i.p. administration (fig. 1 and fig. 2).

The influence of the treatment with fatty acid amide analogues on plasmatic lipid profile and glycaemia

The hypercaloric diet induced in obesity-induced control group a significant increase of the glycaemia and a slight increase of the total cholesterol and triglycerides plasmatic levels comparing to the normal weight mice.

The treatment with **N1**, **N2** and **N3** decreased the glycaemia comparing to the obese group. No significant differences were noticed comparing the groups treated with synthesised fatty acid amide analogues to the normal weight group after 10 days of administration (table 2). The substances may have a potential regulatory effect on the blood glucose level but further studies are required.

The plasmatic lipid profile was improved after the treatment with fatty acid amide analogues (table 2). The plasmatic total cholesterol was significant decreased ($p<0.001$) by 41%, 46% and 60% after the treatment with

Treatment	Glycaemia	Total Cholesterol	Triglycerides
	Mean ±SD	Mean ±SD	Mean ±SD
N1	156.43±22.57	132.27±12.25***	88.66±25.12*
N2	142.32±26.10	121.89±14.40***	74.18±10.95*
N3	157.89±25.88	89.29±17.11***	110.32±19.66
Obese	213.94±50.90*	234.07±45.20	129.48±16.74
Normal	161.50±51.66	226.91±46.68	125.56±13.73

Table 2
THE VARIATION OF THE PLASMATIC BIOCHEMICAL PARAMETERS AFTER THE TREATMENT WITH FATTY ACID AMIDE ANALOGUES

*SD=standard deviation; Statistical significance determined by t-student test: *p<0.05, **p<0.01, ***p<0.001 compared to normal weight control.*

N1, **N2** and respectively **N3**. The blood triglyceride levels were significantly decreased ($p<0.05$) by 30% and 41% in case of **N1** and **N2** administrations. A slight decrease ($p>0.05$) of the triglyceride level was determined in case of **N3** treatment comparing to the control groups. The treatment with fatty acid amide analogues may improve the plasmatic lipid profile in case of hypercaloric diet.

Conclusions

We synthesized three other fatty acid amide analogues, spaced by a CH_2 or CH_2CH_2 linker from the aromatic ring, for preliminary study of their pharmacological activity regarding body weight and food-intake effects. The compounds were obtained by direct amidation of oleic acid, activated with carbonyldiimidazole, with amines: 3-methylpyridinyl, 4-methylpyridinyl and 2-(4-hydroxy-phenyl)ethyl. IR, MS, ^{1}H-, ^{13}C-NMR and complementary APT, COSY and HETCOR spectra confirmed the structure of the fatty acid amide analogues.

The tested compounds decreased the obese animal weight and reduced the food consumption in case of maintaining a hypercaloric diet. Moreover, the 10 days of treatment improved the plasmatic lipid profile and the glycaemia levels. The treatment with the oleic acid amide analogues may have potential pharmacological use in regulating the body weight by regulating the food-intake.

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