Amantadine analogues – synthesis and biological activity

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The biological activity of adamantane derivatives is due to the symmetry and steric bulkiness of the structure and the significant lipophilicity of the rigid hydrocarbon framework. This enables them to penetrate easily through biological membranes. Therefore, modification of organic compounds by an adamantyl radical changes significantly their biological activity, often enhancing it. A large number of strains that are completely resistant to amantadine are currently known. The number of resistant influenza strains increases every year because of spontaneous mutations in the virus genome. This necessitates expanded research on the reasons for the development of resistance and ways of overcoming it by creating new antiviral drugs [1].

Keywords: adamantanes, amino acids, amino acid amides, antiviral activity

INTRODUCTION

Amantadine was a drug that was once used to treat the influenza virus A. The influenza virus A became resistant to amantadine due to the misuse of the drug. In 2005 the drug was used in China as a way to treat the avian influenza. The Chinese gave approximately 2.6 billion doses of amantadine to the chickens, which is what caused it to become resistant [2]. The avian flu threat served as a catalyst for scientists to develop a new antibiotic that could be used to treat drug-resistant bacterial and viral strains [3]. According to Plumb’s Veterinary Drug Handbook, amantadine is safe to use in small animals and equine. It also states that amantadine is still able to treat some influenza viruses, but they have found that the greatest interest in its use is treating chronic pain. The influenza virus A is able to treat is found in equine and is known as equine-2 influenza virus. The handbook states that amantadine is safe for humans and that it is no longer used for fighting the influenza virus but used in Parkinsonian Syndrome [4]. Amantadine is an amino-analogue of adamantane.

Our goal was to modify amantadine with L-Valine, L-Alanine and L-Lys and to investigate their antiviral activity against influenza virus A (H3N2) (Scheme 1). The structures of new analogues were confirmed by NMR and MS analyzes.

EXPERIMENTAL

General information

All amino acids (N-(tert-Butyloxycarbonyl)-L-valine, N-(tert-Butyloxycarbonyl)-L-alanine, N-(tert-Butyloxycarbonyl)-L-lysine and N°α-c.

bis-Boc-L-lysine dicyclohexylammonium salt), amantadine hydrochloride, as well as isobutyl chloroformate (IBCF), triethylamine (TEA), were purchased from Sigma Aldrich (FOT, Bulgaria). Dichloromethane was obtained from Fisher Chemical (Bulgaria) and further was distilled. Chloroform was of reagent grade and used without further purification. Thin-layer chromatography (TLC) was conducted on precoated Kieselgel 60F254 plates (Merck, Germany).

The NMR experiments were recorded on Bruker Avance III 600 or Bruker Avance III 400 spectrometer, operating at 600.13 and 400.15 MHz for protons respectively. The measurements in CDCl3 solutions were carried out at ambient temperature (300 K) and tetramethylsilane (TMS) was used as an internal standard. The UV spectra of the compounds were measured with an “Agilent 8453” UV-vis spectrophotometer. Electrospray ionisation (ESI) and El mass spectra were recorded corresponding on an Esquire 3000 and MAT 8230.

General procedures. Synthesis of 3a-c

The tert-butyloxycarbonyl (Boc) amino acids (Boc-Val-OH, Boc-Ala-OH, Boc-Lys(Boc)-OH) (69 mmol) were dissolved in 2 ml CH2Cl2. The solution was cooled to -15°C, added TEA (69 mmol) and dropwise isobutyl chloroformate (69 mmol). The amantadine (46 mmol) were dissolved in 2 ml mix CH2Cl2/CHCl3 in ratio 1:1, added TEA (46 mmol). After 15 min the solutions were mixed and stirred for 1h at -15°C. The mixture was poured into 5% NaHCO3, extracted with CHCl3, washed with brine, dried over Na2SO4 and concentrated in vacuo. The residues was purified by
The resulting white solid, Boc-Valyl-Amantadine, Boc-Alanyl-Amantadine and oil Boc-Lysyl(Boc)-Amantadine were dissolved in 2 ml of 50% TFA/ CHCl₃ and stirred at 0°C for 1h to remove the Boc group.

Boc-Valyl-Amantadine (Boc-Val-Am) - M.W. = 350.5; 
ESI-MS: 723.4 [M+Na]+, 389.1 [M+K]+, 373.2 [M+Na]+, 351.2 [M+H]+; ¹H NMR (CDCl₃) /600 MHz/ δ (ppm): 0.901 (d, J=6.8 Hz, 3H), 0.937 (d, J=6.5 Hz, 3H), 1.435 (s, 9H), 1.664 (br t, 6H), 1.985 (s, 6H), 2.03 (overlapping, 1H), 2.06 (s, 3H), 3.705 (br t, J= 7.9 Hz, 1H), 5.09 (d, J= 8.2 Hz, 1H), 5.478 (s, 1H).

After removing the Boc group - white powder, 36% yield;
ESI-MS: 523.2 [M+Na]+, 273.1 [M+Na]+

Boc-Alanyl-Amantadine (Boc-Ala-Am) - M.W. = 322,5;
ESI-MS: 667.2 [M+Na]+, 361.1 [M+K]+, 345.1 [M+Na]+, 323.1 [M+H]+; ¹H NMR (CDCl₃) /600 MHz/ δ (ppm): 1.30, (d, J=7Hz, 3H), 1.44 (s, 9H), 1.66 (s, 6H), 1.973 (s, 6H), 2.065 (s, 3H), 4.024 (br s, 1H, CH), 5.024 (br s, 1H, NH), 5.796 (br s, 1H, NH).

After removing the Boc group – white powder, 58% yield;
ESI-MS: 467.1 [M+Na]+, 245.0 [M+Na]+

Boc-Lysyl(Boc)-Amantadine (Boc-Lys(Boc)-Am) - M.W. = 479.7;
ESI-MS: 981.4 [M+Na]+, 502.2 [M+Na]+, 480.3 [M+H]+; ¹H NMR (CDCl₃) /600 MHz/ δ (ppm): 1.44 (s, 18H, Me), 1.40-1.90 (m, 6H, CH₂), 3.11 (br s, 2H, CH₂), 4.13 (br s, 1H, CH), 4.80 (br s, 1H, NH), 5.44 (br s, 1H, NH).

After removing the Boc groups – white to yellow powder, 28% yield;
ESI-MS: 581.8 [M+Na]+, 302.1 [M+Na]+

Viral suspension is drawn from the collection of the "Virology" of the "Stefan Angelov" Institute of Microbiology, Bulgarian Academy of Sciences.

The virus is cultured in a maintenance environment DMEM (Dulbecco's Modified Eagles's Medium) (Gibco BRL, USA) with 0.5% fetal veal serum, 10 mM HEPES (Merk, Germany) and antibiotics (penicillin 100 UI / mL and streptomycin 100 μg / mL) at 37 °C in the presence of 5% CO₂. After seeding in microtitre plates, MDCK cells were incubated at 5% CO₂, 37 °C and 95% humidity for 48 h. Thereafter, the cell culture medium was aspirated and serially diluted compound concentrations in fresh cell culture medium were added (100 μl/well; 2 parallels/concentration, dilution factor 2). Six untreated wells were used as cell control (negative control). 72 h after compound addition and incubation cell were stained with a crystal violet/methanol solution. After dissolving away the stain, the optical density (OD) of individual wells was determined in a Dynatech microplate Photometer (550 /630 nm) and compared with the mean optical density of the 6 cell controls.

RESULTS AND DISCUSSION

We presents test results for the ability of the prepared compounds to inhibit virus A/H3N2 strain Aichi and the cytotoxic activity of the compounds on MDCK cell culture.

Antiviral activity of the compounds was studied at a concentration of 5μg/mL. The percent inhibition of the compounds given in Table 1 is the arithmetic mean.

It can be concluded by analyzing the results that the antiviral activity is depends by the length the carbohydrate chain. There is an optimal size and structure.

As we can see in the table branched chain amino acids valine did not show activity, even it’s hydrophobic portion together with the lipophilic carbocyclic adamantane created the optimum structure for penetrating the membrane bilayer of the virus capsule and disrupting replication processes valine derivative (3 a) behaved no antiviral activity on A(H3N2) virus. Compound 3 b represented amantadine linked to the amino acid alanine. It slightly inhibited A(H3N2). The moderate inhibition activity of this compound possibly is due to its compact structure, which fit into M2 protein envelope and partially obstructs proton conduction.
Table 1. Antiviral activity against influenza virus A (H3N2) and cytotoxicity of the amantadine derivatives.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Cytotoxicity (CC₅₀)</th>
<th>Inhibition (IC₅₀)</th>
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<tbody>
<tr>
<td>Val-Am (3 a)</td>
<td>189.02</td>
<td>0</td>
</tr>
<tr>
<td>Ala-Am (3 b)</td>
<td>211.16</td>
<td>33.28</td>
</tr>
<tr>
<td>Lys-Am (3 c)</td>
<td>206.13</td>
<td>15.32</td>
</tr>
<tr>
<td>Amantadine</td>
<td>&gt; 200</td>
<td>n/a</td>
</tr>
</tbody>
</table>

Based on the activity of the ornithine derivative, which Shibnev and etc. report, we synthesized close structural analog – lysyl-amantadine (3 c). The activity of the compound was slightly less than that of 3 b. Carbohydrate chain is much longer compare to 3 b, but in this derivative are two amino groups, which increase the basic properties and inclination to formation hydrogen bonds with M2 protein [5].

CONCLUSION

We synthesized and tested three compounds of amantadine analogues with the amino acids valine, alanine and lysine. The new analogues 3 a-c were evaluated for their antiviral activity towards influenza virus A(H3N2). This study shows that the antiviral properties of amantadine amino acid derivatives could resuscitation the antiviral properties and the resistance was overcome.

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