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NEW DERIVATIVES OF OSELTAMIVIR WITH BILE ACIDS

[1]Kiril Chuchkov, [1]Silvia Nakova, [2]Lucia Mukova, [2]Angel Galabov, [1]Ivanka Stankova
[1]*South-West University "Neofit Rilski", Blagoevgrad, Bulgaria*
[2]*Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences,Sofia, Bulgaria*

Abstract. Several derivatives of oseltamivir with bile acids (cholic, deoxycholic and chenodeoxycholic acids) were prepared, using N, N dicyclohexylcarbodiimide (DCC) as coupling reagent. The chemical modifications were made in order to enhance the bioavailability of the active substance and combating resistance to the neuraminidase inhibitor - oseltamivir. Moreover, the prepared products were tested for antiviral activity against virus A/Aichi/2/68 (H3N2) and confirmed by plaque reduction assay on MDCK cells. The studied compounds showed no effect against influenza virus A(H3N2).

Keywords: oseltamivir, bile acids, Iinfluenza virus

Introduction

Influenza viruses are respiratory pathogens that affect humans and are responsible for substantial morbidity, mortality and decreased productivity around the world (Hsieh & Hsu, 2007; Yeh et al., 2010). Currently available drugs for the prevention and treatment of seasonal influenza virus infections are the M2 ion channel blockers (amantadine and rimantadine) and the neuraminidase (NA) inhibitors (oseltamivir and zanamivir) (Fig. 1) (De Clercq, 2006). The clinical usefulness of amantadine and rimantadine is limited due to the increasing incidence of adamantane-resistant viruses in the population (Bright et al., 2005, Deyde et al., 2007). Moreover, the M2 ion channel blockers inhibit only influenza A virus replication and are associated with neurological side effects. NA inhibitors are favored clinically, since they are effective against all NA subtypes, are well tolerated, and have a higher barrier for resistance (Moscona, 2008). However, drug-resistant isolates have been detected in A/H3N2- and A/H5N1-infected patients receiving oseltamivir treatment (Kiso et al., 2004).

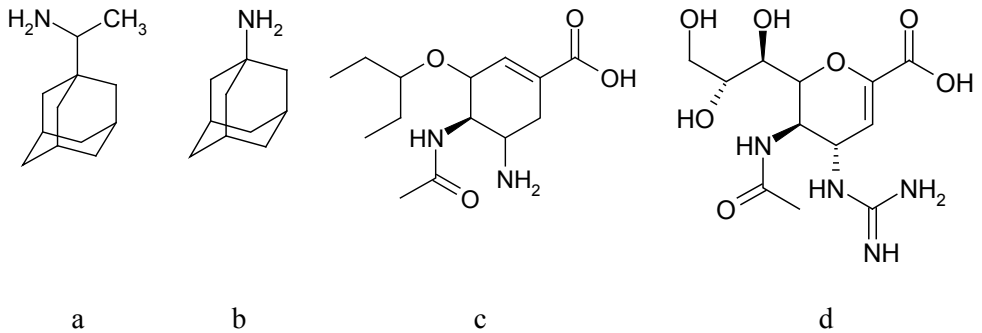


Fig. 1. M2 ion channel blockers amantadine (a); rimantadine (b); neuraminidase (NA) inhibitors oseltamivir (c); and zanamivir (d)

Influenza virus neuraminidase (NA) is thought to promote virus entry and release of virion progeny, thereby enhances infection efficiency. Rationally designed NA inhibitors (NAIs) that block the viral life cycle are proved to be effective for the treatment of influenza. The NAIs have currently emerged as promising therapeutics for influenza. The first approved neuraminidase inhibitor zanamivir (Relenza) is rarely used because it is administered by inhalation, (Von Itzstein et al., 1993) which causes inconvenience. Subsequently discovered oseltamivir (Tamiflu) is orally available (Kim et al., 1997). As the predominant choice, oseltamivir is used worldwide for the treatment of influenza. Peramivir, the third neuraminidase inhibitor, displays only limited oral bioavailability (Bantia et al., 2006; Chand et al., 2005). Intravenous peramivir is currently undergoing a preemergency use authorization review for its use in cases of severe influenza outbreaks.

One of the huge problems of medicine besides the emergence of resistance it turns out and the potential passage of drug through the cell membrane and transfer it to the target cell. (Griffiths & Sjövall, 2010). A Potential to facilitate the passage of biologically active substances through the cell membrane is to connecting them with amphiphilic transport molecules. A good approach to solving this problem is to modify a structure of "molecular umbrella." The "molecular umbrella" contains two or more molecules of bile acid amphiphile which envelop the biologically active substance and facilitates its transition through the cell membrane. Bile acids are harmless molecules since they build up cell membranes of living organisms.

In view of the above and in continuation to our research program concerned with structural modification of certain biologically active compound with the purpose of

enhancing their biological activity and bioavailability. We report here the synthesis of several novel analogues of oseltamivir with bile acids hoping that they could have some chemical and biological interest.

Experimental

The oseltamivir and bile acids were purchased from Sigma, and DMAP and dicyclohexylcarbodiimide hydrochloride (DCC) from Merck. TLC analysis was performed on aluminum silica gel sheets 60 F254 plates (Merck) and spots were detected using an UV lamp at 254 nm. In the column chromatography was used silica gel 60 (230-400) from Merck (Germany). As eluting systems are used chloroform / methanol (8:4).

Synthesis of oseltamivir with bile acids

For the chemical modification of new oseltamivir analogues with bile acids was used carbodiimide method, using DCC as activator, DMAP was used as reaction catalyst.

Oseltamivir-bile acids (2a-c)

In the solution of oseltamivir phosphat 0,150g (48 mmol) in THF was added dropwise to the solution triethylamine – 0.06ml (48 mmol), and stirred at 0°C temperature for 1 hour. In due time in another flask was dissolve in THF (cholic, deoxycholic and chenodeoxycholic acids) (96 mmol) and add N, N-dicyclohexylcarbodiimide (DCC) 0,165 g (80 mmol), the mixture cooling to 0ºC and leave to stir 1h. After 1h two mixtures were combined and adding 4-(N,N-dimethylamino)-pyridine (DMAP) 0.1170g (96 mmol) at 0°C and leave to stir for 24h. Then THF was evaporated in vacuo and the residue was chromatographed on silica gel, using chloroform/methanol (8:4).

Oseltamivircholat (2 a). 1H NMR (400 MHz, in ppm, solvent dmso-d6): 10.65 (br s, 1H, NH), 8.23 (d, 0.5H), 7.78 (s, 1H, H8), 6.48 (br. s, 2H, NH2), 5.56 (0.5H), 5.39 (s, 2H, >N-CH2-O, H1'), 4.45 (br d, J=3.8 Hz, 2H, OH), 4.19 (br s, 2H, OH), 4.16 (m, 0.5H), 4.08 (dd, J= 12.0, 3.0 Hz, 2 x O-CH2 a), 4.02 (m, 1H, O-CH<), 3.96 (dd, J= 12.0, 5.6 Hz, 2 x O-CH2 b), 3.77 (m, 3H, 3 x HO-CH), 3.37 (m, 1H, HO-CH), 2.4-2.0 (m, 6H, 2 x OOC-CH2 , -O-CH2), 1.9-0.9 (m, 70H, choline), 0.9-0.75 (m, 20H, 2 x CH3, 2 x CH choline), 0.58 (9H, 2 x CH3). 13C NMR (400 MHz, in ppm, solvent dmso-d6): 173.0 (2 x –COO), 170.1 (COO), 156.9 (CO), 154.1 (CNH2, C2), 73.8 (OCH(CH2)2), 71.0 (>NCH2O, 70.0 (-CHOH, choline), 62.8 (Cq), 62.0 (Cq), 52.5 (CH), 49.5 (CH), 47.4 (CH), 46.2-46.0 (4Ch), 41.6 (CH), 36.3 (CH2), 35.6 (CH), 35.1 (CH2), 34.8 (CH), 33.8 (Cq), 33.3 (CH2), 32.9 (CH), 32.0-23.3 (18CH2), 23.1 (CH3, choline), 22.8 (CH), 22.1 (Cq), 17.0 (CH3), 16.8 (CH3, choline), 12.4 (CH3, side chains choline).

Oseltamivirdeoxycholat (2 b). 1H NMR (400 MHz, in ppm, solvent dmso-d6): 10.5 (br s, 1H, NH), 7.67 (s, 1H, H8), 6.40 (br. s, 2H, NH2), 4.45 (d, J=4.1 Hz, 2H, OH), 4.18 (br s, 2H, OH), 3.76 (br s, 2H, 2 x HO-CH), 3.37 (br s, 2H, 2 x HO-CH), 2.24 (m, 4H, 2 x OOC-CH2), 1.84-1.39 (m, 20H, choline), 1.39-0.95 (m, 34H, choline), 0.96-0.87 (m, 5H, CH + CH2, choline), 0.83 (s, 12H, 4 x CH3, choline), 0.55 (s, 6H, 2 x CH3, side chains choline). 13C NMR (400 MHz, in ppm, solvent dmso-d6): 137.5 (C8), 70.6 (-CHOH, choline), 69.8 (-CHOH, choline), 62.8 (2 x CHCH2O), 47.6 (CH2), 46.2 (CH2), 40.1 (>NCH2CH2), 30.3 (OOCCH2), 23.2 (16C), 23.8 (CH), 23.1 (CH3, choline), 22.4 (CH), 17.0 (CH3, choline), 12.3 (2 x CH3, side chains choline).

Oseltamivirchenodeoxycholat (2 c). 1H NMR (400 MHz, in ppm, solvent dmso-d6): 10.66 (br s, 1H, NH), 7.78 (s, 1H, H8), 6.54 (br. s, 2H, NH2), 5.39 (s, 2H, >N-CH2-O, H1'), 4.33 (d, J=4.77 Hz, 1H, OH), 4.10 (m, 3H, OH), 4.08-3.90 (m, 5H, 2 x O-CH2 + O-CH<), 3.61 (m, 2H, 2 x HO-CH), 3.16 (m, 2H, 2 x HO-CH), 3.15 (d, J=4.8 Hz, 5H), 2.3-2.0 (m, 6H, 2 x OOC-CH2 , -O-CH2), 2.0-0.9 (m, 44H, choline), 0.84 (m, 2H, CH2, choline), 0.83 (s, 12H, 4 x CH3, choline), 0.58 (s, 6H, 2 x CH3, side chains choline). 13C NMR (400 MHz, in ppm, solvent dmso-d6): 172.0 (2 x –COO), 156.7 (CO), 153.9 (CNH2, C2), 151.3 (NCN, C4), 137.5 (C8), 116.5 (C5), 73.7 (OCH(CH2)2), 71.0 (>NCH2O-), 70.3 (-CHOH, choline), 66.2 (-CHOH, choline), 62.5 (2 x CH2OH), 55.5 (CH), 50.0 (CH), 48.6 (CH), 41.4 (CH), 39.6 (CH2), 39.4 (CH2), 39.2 (CH), 35.3 (CH2), 34.8 (CH + CH2), 32.3 (CH), 30.5 (CH2), 30.3 (CH2), 27.7 (CH2), 23.2 (CH2), 22.7 (CH3, choline), 20.3 (CH2), 18.1 (CH3, choline), 11.6 (2 x CH3, side chains choline).

Antiviral activity

Viruses: Influenza A virus [Aichi/2/68 (H3N2)], from the collection of the Stephan Angeloff Institute of Microbiology, BAS (Sofia, Bulgaria).

Antiviral activity testing

The replication of influenza viruses in MDCK cells induces the complete destruction of host cells, a distinct cytopathic effect (CPE). The virus induced CPE can be inhibited by addition of antiviral compounds (100µl/well;2 parallels/concentration, dilution factor 2). Untreated (virus control) and compo untreated confluent monolayers of test cells were infected with a multiplicity of infection that induces a complete CPE in virus control 24 h after virus addition. Thereafter, adherent cells were fixed and stained with a crystal violet/formalin solution. After elution of the stain, inhibition of virus induced CPE was quantified by optical density (OD) determination in a Dynatech microplate reader. The percentage of antiviral activities of tests compounds was calculated. Based on the mean

dose response curve of at least 2 assays, the 50 % CPE inhibitory concentration (IC50) was calculated.

Determination of cytotoxicity

The maximal tolerated concentration (MTC) is determined as that concentration at which no visible changes in the cell monolayer are observed. The 50% cytotoxic concentration (CC50) is calculated in comparison to the cell control by applying the regression analysis with the help of Origin 6.1 computer program. Cytotoxicity assay CPE (cytopathic effect)-inhibitory assays was quantified by optical density (OD) determination in a Dynatech microplate reader. Cytotoxicity assay for determination of the 50 % cytotoxic concentrations (CC50) of test compounds in MDCK (Madin Darby canine kidney cell monolayers) (Schmidtke et al.. 2001).

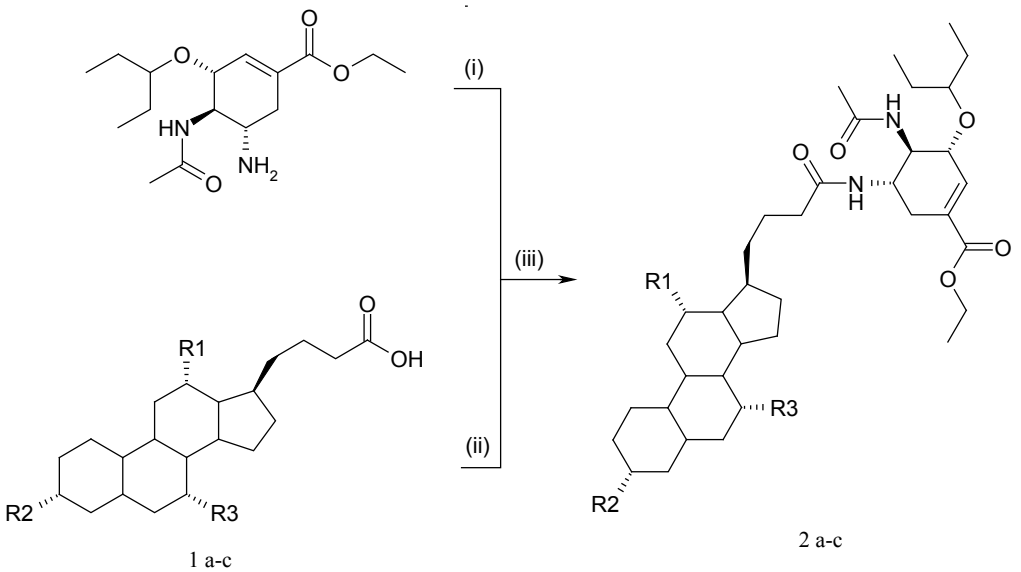
Results and discussion

Some bile acids and their derivatives can be used as drug carriers. A large number of studies have shown that conjugating drugs with appropriate bile acid could increase their enterohepatic absorption, improve metabolic stability, enhance oral bioavailability, and reduce toxicity. The latest research focuses on structure-activity relationships and the application of bile acid as a drug carrier. These studies have indicated that drugs coupled to the bile acid 3 and 24 sites did not affect the acid part of the activity, and the activities of most drugs were not affected by coupling to a bile acid. Therefore, bile acids have the potential to be useful drug carriers (Li et al., 2014).

In our study, oseltamivir-bile acid analogues were prepared using different bile acids: cholic acid (2a), deoxycholic acid (2b), chenodeoxycholic acid (2c), as shown in (Scheme 1). The target compounds (2a-c) were synthesized by forming an amide connecting the bile acid with oseltamivir. The oseltamivir was mixed with triethylamine in THF, bile acids was mixed with N, N-dicyclohexylcarbodiimide (DCC) and a catalyst, 4-dimethylaminopyridine (DMAP), at 0°C and leave to stir for 24 hours to obtain the desired compounds in yields of 75%–76%.

Biological activity

After seeding in microtitre plates, MDCK cells were incubated at 5 % CO2, 37 °C and 95 % humidity for 48h. 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



R1, R2, R3=OH - Cholic acid (a)
R1, R2=OH - Deoxycholic acid (b)
R2, R3=OH - Chenodeoxycholic acid (c)

(i) Et3N, THF, 0°C, 1h (ii) DCC, THF, 0°C, 1h (iii) DMAP, 0°C, 24h

Scheme 1. Synthesis of oseltamivir analogues with bile acids

(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. Here the mean OD of cell controls was put on a level with 100 % vitality of cells. The CC50 as well as the maximal-tolerated concentrations (CC10 = 90% viability of test cells) were calculated from the mean dose-response curve.

Initially the new analogues **2a-c** were evaluated for their antiviral activity towards influenza virus A(H3N2). Results of the antiviral screening of the oseltamivir analogues with bile acids are shown in Table 1. The new analogues of oseltamivir do not show activity.

Table 1. Effect of compounds (2 a-b) on replication of influenza virus A/ Aichi/2/68 (H3N2) in MDCK cells

Compound	CC_{50}[a] μM/L	IC_{50}[b] μM/L Tested	SI[c]
2a	613.9	without effect	-
2b	528.98	without effect	-
2c	569.21	without effect	-

[a]50% cytotoxicity concentration
[b]50% inhibitory concentration
[c]Selectivity index (CC50/ IC50)

Conclusion

Novel oseltamivir analogues have been synthesized with bile acids and their activity on the Influenza virus A Aichi/2/68 (H3N2) have been explored. The studied compounds showed no effect against influenza virus A (H3N2).

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✉ **Dr. Ivanka Stankova** (corresponding author)
Departrment of Chemistry
South-West University Neofit Rilski
66 Ivan Michailov Str.
2700 Blagoevgrad, Bulgaria
E-mail: ivastankova@swu.bg