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AMANTADINE AMIDES PRODRUGS AS HEPATIC DELIVERY SYSTEMS TO ENHANCE ITS ACTIVITY AGAINST HCV

TAREK ABOUL-FADL[a,b], MAHMOUD M. SHEHA[b], ADEL S. EL-AZAB[b], HATEM A. ABDEL-AZIZ[a]

[a]*Department of Pharmaceutical Chemistry, College of Pharmacy, King Saud University, P.O. Box 2457, Riyadh 11451, Saudi Arabia;*

[b]*Department of Pharmaceutical Medicinal Chemistry, Faculty of Pharmacy, Assiut University, Assiut 71526, Egypt.*

To enhance the activity of amantadine against HCV, its amide prodrugs with thiazolidine-4-carboxylic acid derivatives (**6**-**9**) and bile acids (**10** and **11**) were designed and synthesized. *In vitro* kinetic stability of amide prodrugs **8** and **10** were investigated in aqueous buffer solution with variable pH values (1.2, 4.5. 6.8, 7.4. 8.0) and in biological fluids of 90% human plasma and rat liver homogenate at 37°C. *In vivo* release of the parent drug from these prodrug was investigated in mice with the thioazolidine-4-carboxylic acid amide **8** as representative of these delivery systems. Results from the *in vivo* distribution study indicated that the level of amantadine increased significantly in liver from **8** when compared to amantadine itself. The study suggested the synthesized delivery systems is promising carrier to enhance the hepatic bioavailability of amantadine.

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1. Introduction

Since the identification and molecular cloning of hepatitis C virus (HCV) in the late 1980s, it has been estimated that more than 170 million people are infected with the virus. In approximately 80% of infections the virus is able to elude the body's immune response and succeeds in establishing a chronic infection. The number of individuals infected with HCV continues to increase and persistently infected persons are at risk of developing cirrhosis and hepatocellular carcinoma. While prevention of primary infection is possible, vertical transmission of HCV remains a significant problem especially in developing countries. The current standard of care for the treatment of HCV infection is a combination of pegylated interferon and ribavirin (Peg-IFN/RBV). Because of the adverse effects associated with both interferon (IFN) and ribavirin and because Peg-IFN/RBV provides only about a 45~50% sustained virological response (SVR, undetectable HCV RNA for greater than 24 weeks after cessation of therapy) in genotype 1-infected individuals [1, 2].

Amantadine is a relatively inexpensive antiviral drug with activity noted against the flaviviridae family to which the HCV belongs. Although a few early reports documented a good response to amantadine monotherapy, subsequent studies failed to confirm these results [3,4]. Pilot studies have suggested that the addition of amantadine to IFN is effective against HCV. Brillanti *et al*. reported that the combination treatment with IFN, ribavirin and amantadine did reach a relatively high sustained viral eradication rate of 48% [5]. But, it is still debatable if amantadine alone or in combination with IFN-α and ribavirin could improve viral response in patients who failed to respond to previous combination therapy with IFN-α and ribavirin [6].

*Corresponding author: fadl@ksu.edu.sa

One of the goals of effective drug delivery is to control and optimize the localized release of drug at the target site and rapidly clear the non targeted fraction. In the past few years, there has been considerable interest in the development of a delivery system that can release the drug locally in a highly selective fashion. Some of the benefits of this type of delivery system are significant improve in drug performance in terms of efficacy, safety, and improved patient compliance. The various carriers that are being proposed for hepatic-specific drug delivery based on incorporation of the drug to modified bile acid or cysteine derivatives [7]. Accordingly, improvement of the efficacy of antiviral therapy for chronic hepatitis C with amantadine and to minimize the systemic side effects of the drugs can be achieved through using hepatic chemical delivery system [8].

2. Experimental

2.1 Materials and Equipments

Amantadine HCl, L-cysteine, cholic acid and deoxycholic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). 3-(tert-butoxycarbonyl)-thiazolidine-4-carboxylic acid, thiazolidine-4-carboxylic acid, 3-(tert-butoxycarbonyl)-2-methylthiazolidine-4-carboxylic acid and 2-methylthiazolidine-4-carboxylic acid were synthesized according to the reported literature(s) starting from L-cysteine and their physicochemical constants were consistent with those reported [9-11]. All other chemicals used were of commercially available reagent grade and were used without further purification.

Precoated silica gel plates (kiesel gel 0.25 mm, 60G F254, Merck) were used for thin layer chromatography (TLC).

Infrared (IR) Spectra were recorded as KBr disk using Perkin Elmer FT–IR Spectrum BX apparatus at the research center, College of Pharmacy, King Saud University, Saudi Arabia.

Melting points were determined on a Gallenkamp melting point apparatus, and are uncorrected.

NMR Spectra were scanned in $CDCl_3$ on a Bruker NMR spectrophotometer operating at 500 MHz for ^{1}H and 125.76 MHz for ^{13}C at the research center, college of Pharmacy, King Saud University, Saudi Arabia. Chemical shifts are expressed in δ-values (ppm) relative to TMS as an internal standard. D_2O was added to confirm the exchangeable protons.

Mass spectra were measured on Agilent Triple Quadrupole 6410 QQQ LC/MS with ESI (Electrospray ionization) source.

High-performance liquid chromatography-with Ion Trap 6320 MSMS (LC-MS) from Agilent (HPLC-1200 series quaternary pump, solvent selector, degasser, autosampler, column compartment, Ion Trap 6320 Agilent technologies, USA), the system and data integration were handled with Chemstation for LC-MS. HPLC column was Agilent Zorbax Extend C18, 150 mm (length) x 4.6 mm (internal diameter), 5 µm (particle size), 80Å (porosity). Pre-column used was, Agilent Zorbax Eclipsed XDB C18, 4.6 mm×12.5 mm, 80 Å, 5 µm (Agilent Technologies, Palo Alto, CA, USA). The column oven adjusted at 35 °C. The ion-Trap was set as follow: The MS-Ion trap system was calibrated from 15 to 2200 using Agilent tuning mix applying, in sequence, scan calibration, fragmentation calibration, and isolation calibration and defining positive ion-masses; 118.09, 322.05, 622.05, 922.01, 1521.97, 2121.93.

Mobile phase system consists of 90% Methanol, 10% water, 0.5 ml formic acid/1L. Flow rate = 0.4 ml/min. The average retention time of amantadine, 8 and 10 were approximately 2.45, 6.78 and 11.82 min respectively.

2.2 General procedure for the synthesis of amides 8-13

2.2.1 General procedure for the synthesis of amides 6 and 7

To a solution of 2-methyl-3-(tert-butoxycarbonyl)thiazolidine-4-carboxylic acid and/or 3-(tert-butoxycarbonyl)thiazolidine-4-carboxylic acid (5 mmol)) and triethylamine (5 mmol, 0.7 ml) in dichloromethane (25 ml) at 0 ^{0}C, ethylchloroformate (5 mmol, 0.48 ml) was added, the reaction mixture was stirred at the same temperature to 30 minutes. A mixture of 2-adamantyl-amine hydrochloride (5 mmol, 940 mg) and triethylamine (5 mmol, 0.7 ml) was added and the reaction mixture was stirred at room temperature for 2 hours. The solvent was evaporated under reduced

pressure and the residue was chromatographed ($CHCl_3$ and EtOH 95-5% v/v) and/or ($CHCl_3$ and AcOEt 99.5-0.5 % v/v).

*Amide **6**.* White powder in 77% yield; mp= 150-2°C; IR (KBr) νmax/ cm^{-1}: 3332 (NH), 1674-1700 (2C=O); ^1H NMR ($CDCl_3$) δ: 1.39 (s, 9H, $3CH_3$), 1.58 (s, 6H, adamantane-H), 1.89 (s, 6H, adamantane-H), 1.98 (s, 3H, adamantane-H), 3.0-3.2 (m, 2H, thiazolidine), 3.2-4.56 (m, 3H, thiazolidine), 5.75 (s, 1H, NH, D_2O-exchangeable); MS *m/z* (%) 366.1 (M^+, 6.2), 45.3 (100).

*Amide **7**.* White powder in 54% yield; mp= 129-1 °C; IR (KBr) νmax/ cm^{-1}: 3326 (NH), 1690-1670 (2C=O); ^1H NMR ($CDCl_3$) δ: 1.49 (s, 9H, $3CH_3$), 1.57 (s, 3H, CH_3), 1.69 (s, 6H, adamantane-H), 2.01 (s, 6H, adamantane-H), 2.09 (s, 3H, adamantane-H), 3.85-3.95 (m, 1H, thiazolidine), 4.58-4.65 (m, 3H, thiazolidine), 5.64 (s, 1H, NH, D_2O-exchangeable); MS *m/z* (%) 380.5 (M^+, 4.1), 40.1 (100).

2.2.2 General procedure for the synthesis of amides 8 and 9

A solution of compound **6** or **7** (200 mg) in a dichloromethane (5 ml) was stirred with triflouroacetic acid (1 ml) at the room temperature for 3 hours. The solvent was removed under reduced pressure, the residue was washed with water and dried to afford **8** and **9** in quantitative yield.

*Amide **8**.* Mp 163-5 °C; IR (KBr) νmax/ cm^{-1}: 3352, 3288 (2NH), 1655 (C=O); ^1H NMR ($CDCl_3$) δ: 1.7 (s, 6H, adamantane-H), 2.0 (s, 6H, adamantane-H), 2.1 (s, 3H, adamantane-H), 3.07-3.10 (m, 1H, thiazolidine), 3.42-3.45 (m, 1H, thiazolidine), 3.97-4.22 (m, 2H, thiazolidine), 4.22-4.24 (m, 1H, thiazolidine), 5.3 (s, 1H, NH, D_2O-exchangeable); 6.76 (s, 1H, NH, D_2O-exchangeable); MS *m/z* (%) 266.0 (M^+, 5.6), 40.1 (100).

*Amide **9**.* Mp 130-2°C IR (KBr) νmax/cm^{-1}: 3308, 3284 (2NH), 1643 (C=O); MS *m/z* (%) 280.8 (M^+, 22.8), 88.0 (100).

2.2.3 General procedure for the synthesis of amides 10 and 11

To a solution of cholic acid (**4**) and/or deoxycholic acid (**5**), (5 mmol)) in a mixture of dichloromethane and dimethylformamide (25 ml, 1:1), DCC (10 mmol, 2.o6 gm) and 2-adamantylamine hydrochloride **1** (5 mmol, 940 mg) were added, the reaction mixture was stirred at the room temperature for 6-8 hours. The reaction mixture was filtered, the solvent was evaporated under reduced pressure and the residue was chromatographed ($CHCl_3$ and EtOH 98:2 v/v). in 45 & 42% yield, mp=140-2& 123-4 respectively.

*Amide **10**.* Yield 45%; mp= 140-1 °C; IR (KBr) νmax/ cm^{-1}: 3600-3200 (NH+2OH), 1686 (C=O); ^1H NMR ($CDCl_3$) δ: 0.7 (s, 3H, CH_3), 0.93 (s, 3H, CH_3), 1.0 (d, 3H, CH_3), 1.12-2.0 (m, 39H), 2.32-2.36 (m, 1H), 2.47-2.52 (m, 1H), 3.6-3.64 (m, 1H), 3.67-3.74 (m, 1H), 3.9-3.95 (m, 1H), 4.0 (s, 1H), 7.0 (s, 1H, NH, D_2O-exchangeable); MS *m/z* (%) 525.4 (M^+, 1.5), 43.8 (100).

*Amide **11**.* Yield 42%; mp= 123-4 °C; IR (KBr) νmax/ cm^{-1}: 3600-3200 (NH+2OH), 1686 (C=O); ^1H NMR ($CDCl_3$) δ: 0.7 (s, 3H, CH_3), 0.91 (s, 3H, CH_3), 1.0 (d, 3H, CH_3), 1.12-2.5 (m, 39H), 3.44-3.48 (m, 1H), 3.67-3.75 (m, 2H), 3.86 (s, 1H), 3.9-3.98 (m, 1H), 4.0 (s, 1H), 7.0 (s, 1H, NH, D_2O-exchangeable); MS *m/z* (%) 541.4 (M^+, 3.1), 43.9 (100).

2.3 *In vitro* stability studies of amantadine prodrugs 8 and 10.

2.3.1 Stability in aqueous buffer solutions.

A solution of appropriate amantadine prodrug (50 µg/ml) was incubated in buffer with different buffer solutions of pH values (1.2, 4.5. 6.8, 7.4. 8.0) at 37 ± 0.5°C under nitrogen atmosphere. At appropriate time intervals samples (each of 20 µl portion) were taken and chromatographed using the HPLC analysis protocol described in section 2.1. The residual concentrations displayed a pseudo first order rate of hydrolysis. Results are given in table 1.

2.3.2 Stability in biological media

Human blood was obtained from blood bank (King Abdulaziz University (KAU) hospital, Saudi Arabia). Rat blood was obtained from male Sprague-Dawley rats (Animal Care Unit, KAU). Fresh blood was centrifuged immediately at 1800*g* (model 59A Micro-Centrifuge; Fisher Scientific) and 4°C for 5 min and plasma was collected. For stability studies, plasma was diluted to 90% (v/v) with Hank's Buffered Salt Solution (HBSS), pH 7.4, to maintain the pH of the solution during the experiment.

Rat livers were obtained from male Sprague-Dawley rats (Animal Care Unit, KAU). The tissues were blotted to dryness and cut into small pieces after weighing. The tissue pieces were homogenized immediately on ice with ice-cold HBSS (5 ml/1 g of tissue) using a glass homogenizer (15 strokes, pestle/wall clearance 0.25–0.76 mm). Cell debris and nuclei were removed by centrifugation at 10,000*g* and 4°C for 10 min using a model 59A Micro-Centrifuge (Fisher Scientific). The supernatant was collected for stability studies.

The enzymatic stability of the prodrugs was studied in various biological media at 37°C. Each compound (about 10 μM, final concentration) was incubated with the biological matrix for 8 h in a temperature-controlled shaking water bath (40 rpm, 37± 0.5°C). Samples (20 μL) were taken at appropriate time intervals, and the enzymes activity was immediately quenched by adding 180 μL of methanol, centrifuged. Sample (20 μL) was analyzed by HPLC for the released amantadine. Results are given in table 1.

2.4 *In vivo* distribution studies in mice

According to the requirements of the National Act on the usage of experimental animals, the King Abdulaziz University Animal Ethical Experimentation Committee approved all procedures of our in-vivo studies. Two groups, of each of four mice were treated (IG) with a single dose of

amantadine or its prodrug equivalent to 2 mg/kg of amantadine. At constant time intervals, the animals were sacrificed and blood samples were collected from the ocular artery directly after removing eyeball, and were treated to obtain plasma samples. 1 ml plasma samples were diluted with methanol (4 ml), sonicated for 2 min, centrifuged, and supernatant was dried to under nitrogen stream. Then the animals were dissected and livers were removed, rinsed with cold normal saline, blotted dry with a paper towel, weighed, homogenized with ice-cooled methanol (5 ml / 1g liver tissue), and diluted with methanol (5 ml), centrifuged and supernatant was dried to under nitrogen stream. The residue was dispensed with 1m1 methanol for HPLC analysis.

Results are given in table 2.

3. Results and discussion

3.1 Chemistry

The target amide prodrugs (**6-11**) were synthesized according to the protocol described in the experimental section and shown in figure 1. Simply by coupling the amantadine (**1**) with the corresponding activated carboxylic acids (**2-5**). Activation of the carboxylic acids was achieved by two methods. The first method is a simple and mild condition via *in situ* formation of carboxylic–carbonic anhydrides intermediates. Thus, to obtain amides **6** and **7**, 3-(tert-butoxycarbonyl)-thiazolidine-4-carboxylic acid, thiazolidine-4-carboxylic acid (**2)** or 3-(tert-butoxycarbonyl)-2-methylthiazolidine-4-carboxylic acid and 2-methylthiazolidine-4-carboxylic acid (**3**) were reacted with ethylchloroformate in presence of triethylamine (TEA) at 0^{o}C followed by their coupling with **1**. The carbon dioxide evolution provided a driving force for the major pathway to allow a high yield of the reaction product [12]. Amides **8** and **9**, however, were obtained by de-Boc of the corresponding amides **6** and **7** respectively with triflouroacetic. The second activation method was applied to obtain amides **10** and **11** by reacting **1** with cholic acid (**4**) or deoxycholic acid (**5**) in presence of dicyclohexycarbdiamide (**DCC**).

The structures of the target amides were confirmed on the bases of spectral methods of analyses(IR, ^{1}H-NMR and Mass spectrometry). All spectral data are in accordance with the assumed structures.

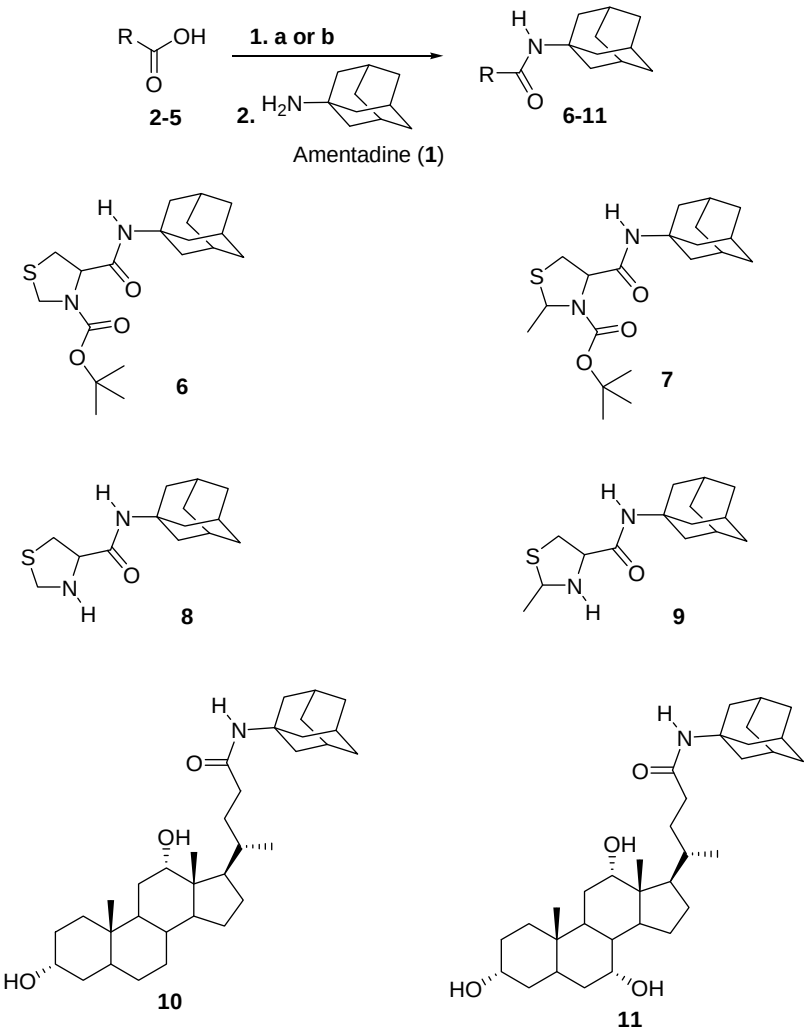
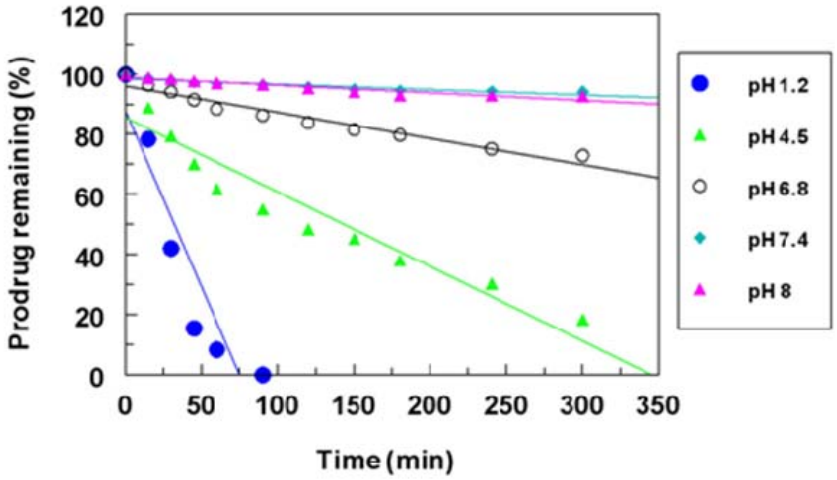


Fig. 1. Synthesis of the target compounds 6-11; a. DCC/DCM, b. TEA/ethylchloroformate/DCM

3.2 *In vitro* kinetic studies of the amide prodrugs 8 and 10

The synthesized amide prodrugs **8** and **10** selected as representatives for thiazolidine-4-carboxilic and bile acid amide derivatives of amantadine to investigate the release profile of the parent drug from these derivatives. The degradation kinetics of **8** and **10** were studied in aqueous buffer solution of pH values 1.2, 4.5. 6.8, 7.4. 8.0 at 37°C. At constant temperature disappearance of the tested compounds displayed strict psudofirst order kinetic reactions, figure 1 and table 1, and all reactions proceeded to completion.

(a)



(b)

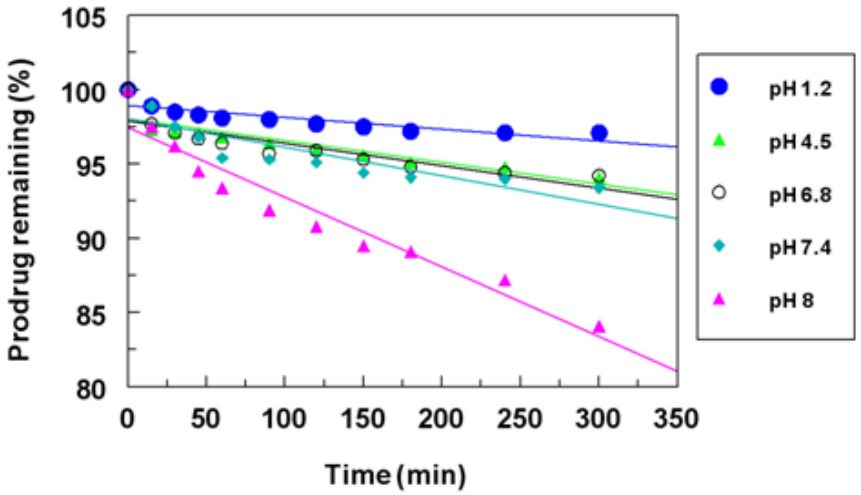


Fig. 2: Psudofirst order degradation plots for the degradation of amide prodrugs 8 (a) and 10 (b) in aqueous buffer solutions of different pH values.

Table 1: Degradation Kinetic data of the synthesized amide prodrugs 8 and 10 in aqueous buffer solutions of different pH values, 90% human plasma and 20% rat liver homogenate at 37 ℃.

Amide Prodrug	K (min^{-1}) pH 1.2	pH 4.5	pH 6.8	pH 7.4	pH 8.0	K (hr^{-1}) 90% Human Plasma	20% Rat Liver Homogenate
8	1.1687	0.2475	0.0872	0.0177	0.0253	19.3700	68.6810
10	0.0078	0.0147	0.0150	0.0191	0.0468	14.2892	48.5016

The rate data obtained for prodrugs **8** and **10** in the aqueous buffer solutions, table 1, revealed that as a general pattern the thiazolidine derivative (**8**) is more susceptible for degradation compared to the deoxycholine derivative (**10**) particularly in acidic medium. This may attributed to the liability of the thiazolidine moiety to chemical decomposition [13].

The rates of the release of parent drug (**1**) from **8** and **10** were also investigated in 90% human plasma and in 20% rat liver homogenate at 37°C. Strict psudofirst order kinetic reactions were also observed with the enzyme systems used under the investigated conditions, table 1.

It is clear from the obtained data that as a general pattern thiazolidine derivative, **8**, is more susceptible toward the investigated enzyme systems compared to the corresponding deoxycholine derivative, **10**. Furthermore, the rate of the release of amantadine from both of the investigated prodrugs was 3.5 times higher in case of rat liver homogenate compared with human plasma, figure 2.

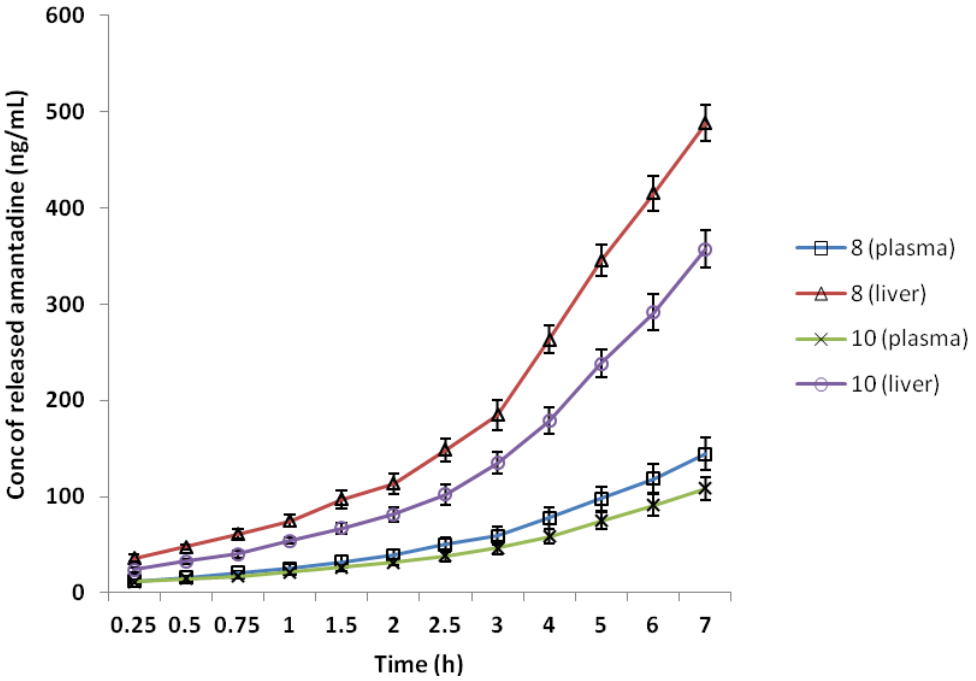


Fig. 3: Release of Amantadine from amide prodrugs 8 and 10 in human plasma and rat liver homogenate under the physiological conditions. Error bars represented the mean and standard deviation of three independent experiments.

3.3 In vivo pharmacokinetic studies of amide prodrug 8

To study the release of amantadine by the prodrug *in vivo*, amide prodrug **8** and the parent drug **1** were given by IG route to mice with a single dose equivalent to 2 mg/kg body weight of amantadine. Blood and livers were collected to analyze the concentration of amantadine at different intervals as described in the experimental section. To understand the *in vivo* behavior of the tested prodrug (**8**), the plasma pharmacokinetics of amantadine and the designed prodrug (**8**) in mice were assessed (Fig. 3). The curves displayed that amantadine could be quickly cleared from circulation while the prodrug showed certain stability which would increases the chance to be transported to the liver. Concentration of amantadine available in blood after administration of parent drug was markedly more than that in the liver when compared at same time. In advantageous its concentration released from prodrug 8 in both blood and liver was approximately comparable during time of study. These finding suggest that prodrug 8 was delivered and accumulated in the liver where the site of metabolism and release of amantadine. The slowly declined concentrations in plasma could be explained by that decomposition of the prodrug **8** extended the metabolism course of amantadine. Concentrations of amantadine in plasma and liver after administration of the parent drug and the prodrug **8** were outlined in fig. 3. Although the regeneration of amantadine seemed not fast, the concentration increased steadily as time went by,

which might indicate sustained release of amantadine by the prodrug in the liver. Moreover, amide prodrug 8 showed satisfactory release of the amantadine in both blood and liver during the investigated course of time compared with amantadine itself.

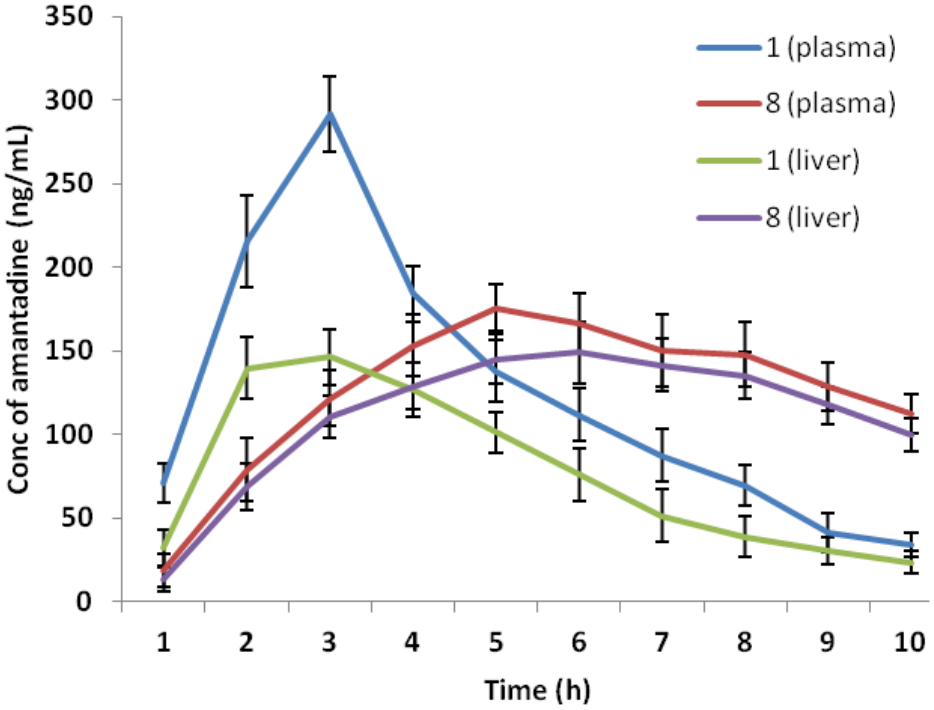


Fig. 3. Concentration curve of amantadine in plasma and liver versus time after administration of Amantadine and the prodrug 8. Error bars show the value of SD (n=3).

In vivo results displayed that designed prodrug **8**, could enhance the delivery of amantadine into liver. Therefore, the designed amide prodrugs will exhibit excellent delivery ability to the liver with lower systemic availability and hence lower systemic toxicity.

4. Conclusion

Amide prodrugs of Amantadine with Thiazolidine-4-carboxylic acids and bile acids were designed and synthesized as chemical delivery systems to liver. Generally, the increased distribution of amantadine in liver after IG administration of **8** suggested that these delivery systems could act as a vector, transporting the drugs to the liver, and beyond which, the designed prodrugs could constantly release amantadine in the liver.

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