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Research Article

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DESIGN, SYNTHESIS AND KINETIC STUDY OF COUMARIN-BASED TRIPLE MUTUAL PRODRUG FOR LUNG CANCER

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ABSTRACT

Owing to our interest in the coumarin-based prodrug system, a novel prodrug of 5-fluorouracil, leucovorin and 5,7-dimethoxy-4-phenylcoumarin was designed, synthesized and evaluated as a promising chemotherapeutic agent for lung cancer depending on *in-vitro* stability study in HCl buffer (pH 1.2) and in phosphate buffer (pH 7.4), as well as in vitro release study in human serum. The synthetic plan was designed to be a modification to that described by Wang et al, and was consist of a series of 7 linear steps starting from 5,7-dimethoxy-4-phenylcoumarin. This coumarin derivative was reduced to diol with relatively low yield; the allylic hydroxyl group was selectively protected via ether formation whereas the phenolic hydroxyl group was esterified with folinyl chloride. When the protective group was cleaved in an acidic medium, the resulted allylic hydroxyl group was selectively oxidized to aldehyde using a selective oxidizing agent, manganese oxide. The allylic carboxylic group resulted from aldehyde oxidation by sodium chlorite was coupled with 5-flourouracil using dicyclohexyl carbodiimide to form the target prodrug. The chemical structure of prodrug was established by analyzing its FTIR, 1H NMR, 13C NMR and MS-ESI spectra. The results of *in-vitro* kinetic study indicated that the prodrug was significantly stable in both buffers with half-lives of about 36hr and 20hr respectively, and was hydrolyzed in human serum followed pseudo first order kinetics with half-life of about 8hr. Consequently, it is believed that the synthesized prodrug may be a potential candidate as oral prodrug for treatment of lung cancer, and is a first agent belongs to a new prodrug strategy, which is a coumarin-based triple mutual prodrug.

Keywords: Coumarin; prodrug; 5-fluorouracil; leucovorin; kinetics.

INTRODUCTION

Non-small cell lung cancer (NSCLC) is a heterogeneous disease that is hard to treat, and remains the leading cause of cancer-related mortality worldwide[1]. In recent years, despite the recent advances in surgery, irradiation and targeted chemotherapy; the survival rate of patients with advanced stage NSCLC is very low (about five years)[2].

The synthetic fluoropyrimidine, 5-Fluorouracil (5-FU), has a broad spectrum of activity against solid tumors as colorectal, breast, gastric, pancreatic, prostate, bladder and lung cancers[3]. However, 5-Fu has heavy toxic side effects including little affinity to tumor cells, a short plasma half-life (so it is administered by intravenous infusion) and irregular absorption with unpredictable plasma level after oral use[4].

As a pyrimidine analog, 5-FU is converted intracellularly to several active metabolites: 5-fluorodeoxyuridine triphosphate (5-FdUTP), 5-fluorodeoxyuridine monophosphate (5-FdUMP) and 5-fluorouridine triphosphate; these active metabolites disrupt RNA synthesis and the action of thymidylate synthase (TS)[5]. Although a host of compounds has been suggested to enhance the clinical effects of 5-FU[6-8], the most intensively studied and widely used biochemical modulator is leucovorin (LV, folinic acid, 5′-formyltetrahydrofolate)[9].

LV improves the inhibitory effect of 5-FU (more precisely of 5-FdUMP) on TS, which is the key enzyme in the *de novo* synthesis of thymidine and the most important target of 5-FU in cancer cells. LV is transported through the cell membrane by the reduced folate carrier and anabolized to methylene tetrahydrofolate (CH_2-THF), the increased intracellular concentration of which results in expansion of the cellular concentration of CH_2-THFand stabilization of its ternary complex with TS and 5-FdUMP[10-12].

Additionally, polyglutamates of CH_2-THF that are formed by the action of folylpolyglutamate synthase may enhance the inhibition of TS[13]. According to huge number of *in vivo* studies, the potentiation of antitumor activity of 5-FU by LV is usually associated with prevention of rebound TS induction and with a more significant inhibition[14-20].

4-Aryl coumarins (neoflavones) are known to exhibit several important biological activities such as antitumor, antimalarial, antibacterial, anti-inflammatory, anti-HIV, antidiabetic, antiviral and antiprotozoal[21]. 5,7-dimethoxy-4-phenylcoumarin, isolated from endophytic *Streptomyces aureofaciens* CMUAc130, showed a significant *in-vivo* antitumor activity against NSCLC. Additionally, it may be effective in preventing or in delaying the formation of

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metastases and may exhibit low toxicity to normal cells[22, 23].

This work reported the use of coumarin-based prodrug system to synthesize a novel prodrug that, on hydrolysis, releases three active moieties: 5-FU, LV and 5,7-dimethoxy-4-phenylcoumarin, and to evaluate it as a promising oral chemotherapeutic agent by studying its *in-vitro* stability in HCl buffer (pH 1.2) and in phosphate buffer (pH 7.4), as well as *in-vitro* release in human serum.

EXPERIMENTAL

Materials

All chemicals and solvents used in this work were purchased from commercial sources and used without further purification. Ethyl benzoylacetate, $LiAlH_4$ and 3,5-dimethoxyphenol were purchased from Tokyo Chemical Industry (TCI) and the others from Fluka.

Instruments

The instruments used for structure identification of the prodrug were: Bruker Avance DRX-400 MHz (Germany) to scan NMR spectra that were expressed in part per million upfield to TMS as an internal standard, Shimadzu LCMS-2020 Single Quadrupole Liquid Chromatograph Mass Spectrometer (Japan) with electrospray ionization source to measure the mass spectrum, Bruker-Alpha ATR-FTIR spectrophotometer (Germany) to record the IR spectrum.

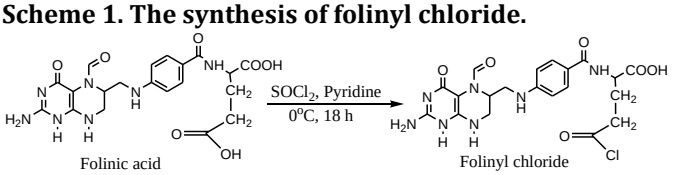
Melting points were determined on an electrochemical CIA 9300 melting point apparatus (UK) using open capillary method and were uncorrected. The instrument used to identify UV spectra and to follow kinetic study was Carrywinn UV Varian UV/Visible spectrophotometer.

The purity of compounds and the completion of reactions were checked by thin layer chromatography (TLC) using precoated silica gel plates (60G F254, Merck) and the spots on chromatograms were localized via UV light (at 366 nm).

Synthesis

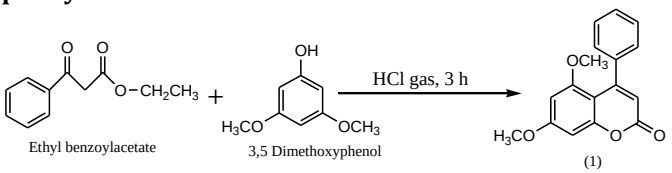
Folinyl chloride: A solution of folinic acid (11.83 g, 25 mmol) in 100 ml freshly distilled DMSO and pyridine (2 ml) was placed in an ice bath. To this solution, freshly distilled thionyl chloride (2 ml, 27 mmol) in 10 ml dry $CHCl_3$ was added drop wise with continuous stirring under anhydrous condition. Then the reaction mixture was stirred in an ice bath for 18 hours and the progress of reaction was monitored by TLC using n-hexane: ethyl acetate (1:1) mixture as mobile phase. Finally the reaction mixture was concentrated to dryness in rotary evaporator; the residue was then re-dissolved in 50 ml ACN, filtered and the filtrate was evaporated to afford the desired product as white creamy powder (Scheme **1**). The melting point was (212-215°C), the percentage of yield was 68% and λ_{max} (ethanol) =273 nm.

Scheme 1. The synthesis of folinyl chloride.



5,7-Dimethoxy-4-phenylcoumarin (1): Dry hydrogen chloride gas was passed through a solution of 3,5-dimethoxyphenol (7.7 g, 0.05 mole) and ethyl benzoylacetate (9 ml, 0.052 mole) in absolute ethanol (50 ml) for 3 hours under anhydrous conditions. The mixture was kept for two days at room temperature and then was kept in refrigerator for 24 hours to complete precipitation. The separated product was filtered, washed with cold ethanol and crystallized from methanol (Scheme **2**).

Scheme 2. The synthesis of 5,7-Dimethoxy-4-phenylcoumarin



(Z)-2- (3-hydroxy -1- phenylpropenyl)- 3,5-dimethoxy phenol (2): A solution of 1 (3.53 g, 12.5 mmol) in 25 ml dry ether was placed in an ice bath and then treated with a solution of 0.5 M of pure $LiAlH_4$ in dry ether (0.95 g of $LiAlH_4$ dissolved in 25 ml, 25 mmol). After stirring for 15 min, 5 % HCl (12.5 ml) was added to the reaction at 0°C. Then the solution was adjusted to pH 5 with 1M HCl and extracted with ether (3×30 ml). The ether layer was dried over Na_2SO_4, filtered and evaporated. The residue was dissolved in ethanol, filtrated and evaporated to afford the desired product. (Scheme **3**)

(Z)-2- [3-(*tert*- butyldimethylsilyloxy)-1-phenyl propenyl]-3,5 dimethoxy phenol (3): A solution of compound 2 (3.26 g, 11.4 mmol) in 40 ml dry THF was placed in an ice bath. To this solution, *tert*-butyldimethylsilyl (TBDMS) chloride (1.9 g, 12.55 mmol) dissolved in 35 ml dry THF was added at 0°C. Then N,N-dimethylaminopyridin (DMAP) (2.09 g, 17 mmol) in 40 ml dry THF was added in a dropwise manner. After stirring for 20 hours at 0°C, the solution was filtered and evaporated to remove the THF. The residue was re-dissolved in ethyl acetate (50 ml) and washed with 1 M HCl (2 × 15 ml), 5 % $NaHCO_3$ (15 ml) and H_2O (15 ml). The ethyl acetate layer was dried over Na_2SO_4, filtered and evaporated. The desired product was then crystallized from a mixture of HCl_3: diethyl ether (1:1). (Scheme **3**)

(Z)-2-[3- (*tert*-butyldimethylsilyloxy) phenylpropenyl] -3,5-dimethoxy phenyl folinate (4): To a solution of compound 3 (2 g, 5 mmol) in 25 ml dry THF treated with K_2CO_3 (600 mg), solution of folinyl chloride (2.46 g, 5 mmol) in 75 ml dry THF was added dropwise for 30 minutes. The reaction mixture was stirred for 30 hours at room temperature. The progress of the reaction mixture was monitored by TLC using ether: ethyl acetate (1:1) mixture as mobile phase. The reaction mixture was then concentrated in rotary evaporator to dryness and the residue was extracted in ethyl acetate. The ethyl acetate layer was washed with water (3×25 ml), dried over anhydrous Na_2SO_4 and evaporated to afford the desired product. (Scheme **3**)

(Z)-2-(3 -hydroxy-1- phenylpropenyl)-3,5- dimethoxy phenyl folinate (5): To a solution of compound 4 (2.57 g, 3 mmol) in THF (15 ml), water (15 ml) was added. This was followed by dropwise addition of acetic acid (45 ml). The mixture was stirred at 0°C for 6 hours and then evaporated to remove THF, water and acetic acid under reduced pressure. Ethyl acetate (100 ml) was added to the residue, which was washed with 5 % $NaHCO_3$ (2 × 50 ml) and water (2 × 50 ml). The ethyl acetate layer was dried over Na_2SO_4, filtered, and evaporated. The desired product was then crystallized from ethanol. (Scheme **3**)

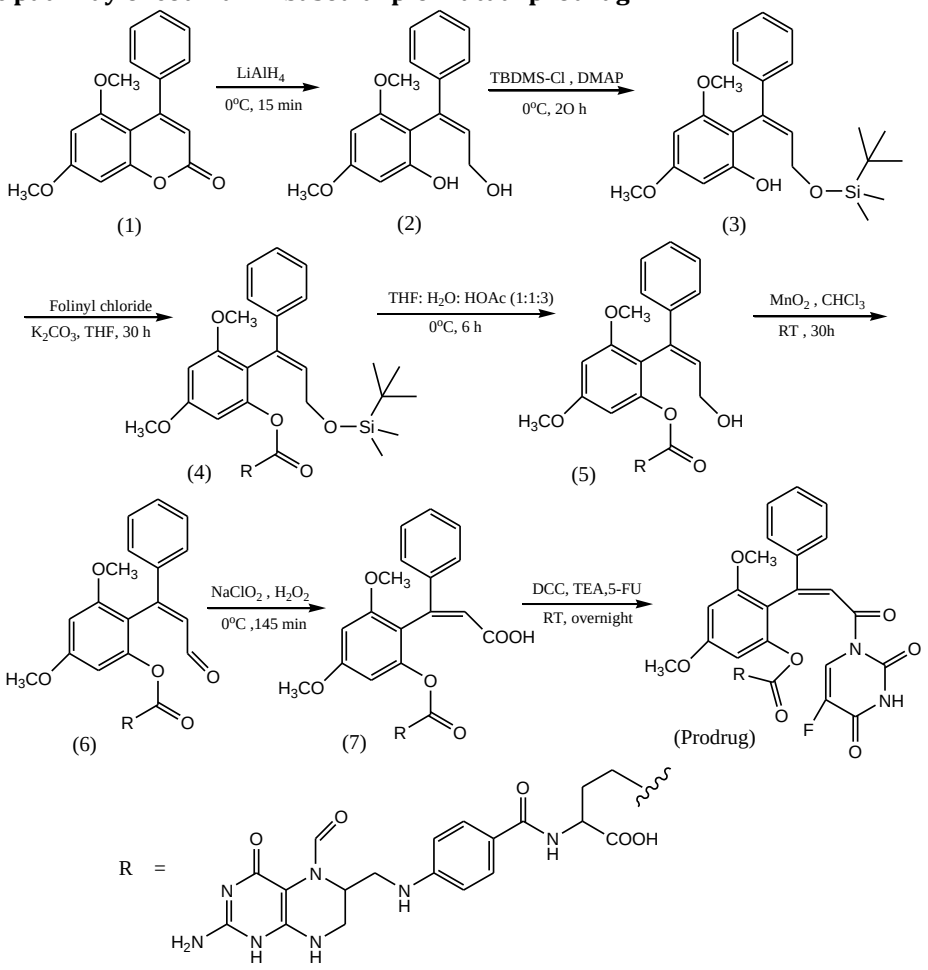
(Z)-2-(3-oxo-1-phenylpropenyl)-3,5- dimethoxyphenyl folinate (6): A suspension of 5 (1.48 g, 2 mmol) and MnO_2 (0.87 g, 10 mmol) in $CHCl_3$ (40 ml) was stirred at room temperature. The resulting suspension was filtered after 30 hours and the filtrate was concentrated to dryness by rotary evaporator. The residue was re-dissolved in 40 ml

ACN, filtered and the filtrate was evaporated to afford the desired product. (Scheme **3**)

(Z)-3-[2- folinyloxy-4,6- dimethoxyphenyl]-3- phenyl acrylic acid (7): A solution of sodium chlorite (330 mg, 3.67 mmol) in water (3 ml) was added dropwise very slowly to a stirred mixture of compound 6 (1.48 g, 2 mmol) in ACN (25 ml), sodium dihydrogen phosphate (35 mg, 0.29 mmol) in water (1.50 ml), and 30 % hydrogen peroxide (1.13 ml, 9.4 mmol). During the addition, the reaction temperature was kept at 0°C in an ice bath and oxygen evolution from the solution was observed visually until the end of the reaction. When oxygen evolution was ended (about 145 minutes from the first addition of sodium chlorite), a small amount of sodium sulfite (0.04 g) was added to destroy the unreacted HOCl and H_2O_2. The solution was acidified with 1 M HCl to pH 2. The mixture was then extracted with ethyl acetate (2×50 ml). The combined ethyl acetate layer was washed with saturated sodium chloride solution (2 × 25 ml), dried over anhydrous Na_2SO_4, filtered and evaporated. The residue was re-dissolved in ACN, treated with aqueous solution of 10% $NaHCO_3$ to pH 6.5 and filtered. The filtrate was acidified with 1M HCl to pH 2.5 and the desired product obtained by filtration. (Scheme **3**)

(Z)-2-[3- (5-fluorouricyl) -3-oxo -1-phenylpropenyl]-3,5- dimethoxy-phenyl folinate (8, prodrug). To a cold solution of 7 (0.76 g, 1 mmol) in 15 ml freshly distilled DMSO, DCC (0.21 g, 1 mmol) in 5 ml freshly distilled DMSO was added and the reaction mixture stirred for 30 minutes. To this solution, a mixture of 5-FU (0.13 g, 1 mmol) and triethylamine (0.3 ml, 2 mmol) in 10 ml freshly distilled DMSO was added in dropwise manner. Reaction mixture was stirred in the dark at 0°C for two hours and then at room temperature for overnight. Precipitated dicyclohexylurea (DHU) was filtered and the solvent was removed at reduced pressure. The dried product was dissolved in 40 ml $CHCl_3$ and filtered to remove the remaining of DHU. The organic layer was washed with 20 ml aqueous solution of 5% sodium bicarbonate, 20 ml saturated NaCl solution and 20 ml distilled water. The organic layer was dried over anhydrous Na_2SO_4, filtered and evaporated. The desired product was crystallized from ACN. (Scheme **3**)

Scheme 3. Synthetic pathway of coumarin-based triple mutual prodrug.



Kinetic study

The synthesized prodrug was exposed to chemical hydrolysis (using buffers of physiological pH values) and enzymatic hydrolysis (using human serum). These hydrolytic reactions were followed by double beam UV/Visible spectrophotometer for decreasing the prodrug concentration with the time.

Stability study in acidic buffer (pH 1.2) and in basic buffer (pH 7.4)

The *in-vitro* chemical hydrolysis of the prodrug was studied in (0.1 M) hydrochloric acid buffer (pH 1.2) and in (0.1 M) phosphate buffered saline (pH 7.4). A sample (5µmol) of prodrug was dissolved in 2 ml anhydrous DMSO in a 100ml beaker. To this solution, 48 ml preheated buffer solution was added with gentle stirring to achieve a final concentration of 100 µM. At the end of addition, the time was detected and the resulted solution was kept at a constant temperature (37 ± 1°C) in a water bath. Then, the solution was divided into a set of ten test tubes; each one would contain 5 ml.

At selected time intervals of 30, 60, 90, 120, 150, 180, 210 and 240 minutes, a test tube was taken from a water bath and its content extracted with 2 ml CH_2Cl_2. Aliquot (2 ml) was withdrawn from aqueous layer and estimated at

defined λ_{max} on UV/Visible spectrophotometer to detect the remaining concentration of prodrug.

Release study in serum. The *in-vitro* enzymatic hydrolysis of prodrug was studied in human serum; a sample (2.5µmol) of prodrug was dissolved in 2 ml phosphate buffered saline in a 50ml beaker. To this solution, 23 ml preheated serum was added with gentle stirring to achieve a final concentration of 100 µM. At the end of addition, the time was detected and the resulted solution was kept at a constant temperature (37 ± 1°C) in a water bath. Then, the solution was divided into a set of ten test tubes; each one would contain 2.5 ml.

At selected time intervals of 30, 60, 90, 120, 150, 180, 210 and 240 minutes, a test tube was taken from a water bath and its content extracted with 2 ml CH_2Cl_2. Aliquot (2 ml) was withdrawn from aqueous layer and estimated at defined λ_{max} on UV/Visible spectrophotometer to detect the remaining concentration of prodrug.

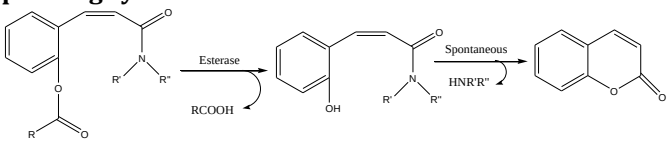
RESULTS AND DISCUSSION

In traditional, 5-FU has been widely used in the treatment of NSCLC, but the severe side effects and the drug resistance limited its clinical usefulness[24]. In an attempt to optimize the antitumor activity of 5-FU against NSCLC, the suggested approach is the use of coumarin-based prodrug system.

This system has many advantages: *first*, it may be used to improve the low bioavailability resulted from low permeability through biological barriers as blood brain barrier and intestinal barrier[25]. To date, this system was successfully used to prepare several prodrugs of opioid peptide[26], non-peptide analgesic[27] and peptidomimetic[28].

Secondly, the system has a *cis*-double bond which could facilitate the lactonization process (Scheme **4**) when an acyl group (R) is released by esterase[29]. *Thirdly*, the release rate from such system can be manipulated by introducing of specific substituents on the aromatic ring or on acyl group[30]. Finally, coumarin, the final released product, is well-known to be non-toxic in extensive studies[31].

Scheme 4. Lactonization process of coumarin-based prodrug system.



In this work, the last advantage has been modified by replacing coumarin as a central nucleus with one of its derivative, which is 5,7-dimethoxy-4-phenylcoumarin. Based on *in-vitro* kinetic profile, the synthesized prodrug was enzymatically released three active moieties: 5-FU, its metabolic modulator (LV) and 5,7-dimethoxy-4-phenylcoumarin, which has antitumor activity against NSCLC through mechanism of action differs from that of 5-FU.

Synthetic part

The prodrug was synthesized through a sequence of 7 linear steps starting from 5,7-dimethoxy-4-phenylcoumarin; this synthetic pathway (scheme **3**) can be considered as a modification to that described by Wang *et al*[32]. The first step involved reduction of substituted coumarin to an open-ring diol with lithium aluminum hydride ($LiAlH_4$). Higher temperature than 0°C and/or longer reaction time may lead to reduce the exocyclic double bond whereas the use of commercially available $LiAlH_4$ may lead to lower the yield intensively.

The following steps involved selective protection of allylic hydroxyl group resulted from the previous step with TBDMS-chloride, and acylation of free phenolic hydroxyl group with folinyl chloride. When aromatic ester is formed, the TBDMS ether was cleaved under acidic condition to afford allylic hydroxyl group that oxidized to carboxylic acid group in two steps. Coupling of the free carboxylic acid with 5-FU was carried out by using dicyclohexyl carbodiimide (DCC) as an activating agent.

Physicochemical properties of compounds 1-8 are shown in table **1** and table **2**.

Table 1. The physicochemical properties of compounds (1-8).

Compound	Physical appearance	% Yield	M.P. (°C)	R_f chloroform: acetone (4:1)	λ_{max} (nm)
1	colorless crystals	53	166-168	0.813	325
2	White crystals	34	193-195	0.508	293
3	White crystals	68	177-179	0.569	289
4	White powder	59	248-252	0.416	339
5	White creamy crystals	62	266-270	0.367	328
6	White powder	66	281-285	0.307	339
7	White creamy powder	69	299-303	0.288	333
8, Prodrug	White creamy crystals	40	268-272	0.373	342

Table 2. The calculated molecular formula, molecular weight and elemental analysis of compounds (1-8).

Compound	Molecular formula	M. Wt.	Elemental analysis					
			%C	%H	%F	%N	%O	%Si
1	$C_{17}H_{14}O_4$	282.29	72.33	5.00	-----	-----	22.67	-----
2	$C_{17}H_{18}O_4$	286.32	71.31	6.34	-----	-----	22.35	-----
3	$C_{23}H_{32}O_4Si$	400.58	68.96	8.05	-----	-----	15.98	7.01
4	$C_{43}H_{53}N_7O_{10}Si$	856.01	60.33	6.24	-----	11.45	18.69	3.28
5	$C_{37}H_{39}N_7O_{10}$	741.75	59.91	5.30	-----	13.22	21.57	-----
6	$C_{37}H_{37}N_7O_{10}$	739.73	60.08	5.04	-----	13.25	21.63	-----
7	$C_{37}H_{37}N_7O_{11}$	755.73	58.80	4.93	-----	12.97	23.29	-----
8, Prodrug	$C_{41}H_{38}FN_9O_{12}$	867.79	56.75	4.41	2.19	14.53	22.12	-----

The structure of compounds (1-8) was confirmed by monitoring the presence and/or absence of specific functional groups using the FTIR spectrophotometer, whereas the structure of prodrug was established by analyzing its FTIR, ^1H NMR, ^{13}C NMR and mass spectra.

^1H-NMR (DMSO-d_6) spectrum of the prodrug showed the chemical shift of the following protons: δ 12.86 ppm (s, 1H, COO***H***), δ 11.95 ppm (s, 1H, O=C-N***H***-C=O), δ 10.30 ppm (s,

1H, NCO***H***), δ 8.92 ppm (s, 1H, CH_2N***H***), δ 8.21 ppm (s, 1H, CHN***H***CO), δ 7.96 ppm (d, 1H, CF=C***H***), δ 7.61 ppm (d, 2H, O=C-C***H***, aromatic), δ 7.2-7.4 ppm (m, 5H, Ar-***H***), δ 7.0 ppm (s, 1H, NH_2-C-N***H***), δ 6.67 ppm (d, 2H, NH-C***H***, aromatic), δ 6.56 ppm (s, 1H, Ar-C=C***H***), δ 6.30 ppm (s, 1H, Ar-N***H***), δ 6.10 ppm (s, 1H, OCH_3-C-C***H***), δ 6.06 ppm (s, 1H, OCH_3-C-C***H***-CO, aromatic), δ 6.0 ppm (s, 2H, N***H₂***), δ 4.85 ppm (m, 1H, CH_2-C***H***-NCHO), δ 4.46 ppm (m, 1H, C***H***-COOH), δ 3.96 ppm (s, 3H, OC***H₃***), δ 3.75 ppm (s, 3H, OC***H₃***), δ 2.7-3.4 ppm (m, 4H, NH-C***H₂***-CH-C***H₂***), δ 2.28 ppm (t, 2H, CO_2-C***H₂***), δ 1.8-1.95 ppm (m, 2H, CO_2-CH_2-C***H₂***).

^{13}C-NMR (DMSO-d_6) spectrum of the prodrug reported the chemical shift of the following carbons: δ 174.74 ppm (***C***OOH), δ 170.07 ppm (O-***C***O-CH_2), δ 167.04 ppm (Ar-***C***O-NH), δ 163.09 ppm (=CH-***C***O-N-CO), δ 161.97 ppm (-***C***O-N=C), δ 160.91 ppm (CF-***C***O-), δ 158.54 ppm (N-***C***HO), δ 158.26 ppm (-***C***H-OCH_3), δ 156.54 ppm (-***C***H-OCH_3), δ 155.39 ppm (Ar-***C***-Ar), δ 153.10 ppm (***C***-NH_2), δ 152.76 ppm (NH-***C***-NH), δ 151.75 ppm (***C***-NH-CH_2), δ 148.54 ppm (N-***C***O-NH), δ 138.49 ppm (***C***F), δ 138.19 ppm (Ar-C-***C***H), δ 129.06, 127.94, 127.31, 127.11, 121.64, 111.51, 95.79 and 93.51 ppm (aromatic carbons), δ 124.59 ppm (FC=***C***H), δ 121.64 ppm (-***C***-CO-NH), δ 112.66 ppm (Ar_2-C=***C***H-), δ 103.50 ppm (-***C***-C-Ar), δ 55.69, 55.40 ppm (O-***C***H_3), δ 52.57 ppm (-***C***H-COOH), δ 42.43 ppm (Ar-NH-***C***H_2-), δ 42.19 ppm (-CH_2-***C***H-CH_2-), δ 41.29 ppm (-CH-***C***H_2-NH-), δ 31.23 ppm (-O-CO-***C***H_2-) and δ 26.86 ppm (-CH_2-***C***H_2-CH-).

FTIR spectrum of the prodrug revealed the characteristic absorption band for the following functional groups: ν 1057 cm^{-1} (C-F), ν 1257 cm^{-1} (Ar-O-CH_3), ν 1674 cm^{-1} (C=O, amide), ν 1731 cm^{-1} (C=O, ester), ν 1759 cm^{-1} (C=O, carboxylic acid), ν 2674 cm^{-1} (O-H, carboxylic acid), ν 2891 cm^{-1} (-CH), ν 3063 cm^{-1} (=CH), ν 3111 cm^{-1} (N-H, amide) and ν 3305 cm^{-1} (N-H, amine).

MS-ESI spectrum (*m/z*) of the prodrug operated in a positive mode characterized the mass of the following products: 868 $[M+H]^+$, 882 [M+Nebulizer gas,CH_3], 890 $[M+Na]^+$, 849 [M- H_2O], 248 [M- (H_2O & CO)].

Kinetic part

Chemical stability: The fundamental requisites for oral delivery prodrug involve its chemical stability at pH values simulating that of physiological fluids and its ability to readily release the active drug(s) after absorption. Consequently, the kinetics of synthesized prodrug was monitored in aqueous buffer solutions of pH 1.2 and pH 7.4. These reactions were studied for the decrease in prodrug concentration versus time utilizing double beam UV/Visible spectrophotometer, and were showed a significant stability of prodrug in both buffers with half-lives of about 36hr and 20hr respectively.

Enzymatic hydrolysis: The prodrug showed encouraging rate of hydrolysis in human plasma (pH 7.4) to regenerate LV, 5-FU and 5,7-dimethoxy-4-phenylcoumarin followed pseudo first order kinetics (Figure **1**) with half-life of about 8hr. Data obtained from kinetic study (average of three trials) were listed in Table **3**, whereas the kinetic parameters listed in Table **4**.

Figure 1. Pseudo first order slope of the *in-vitro* enzymatic hydrolysis.

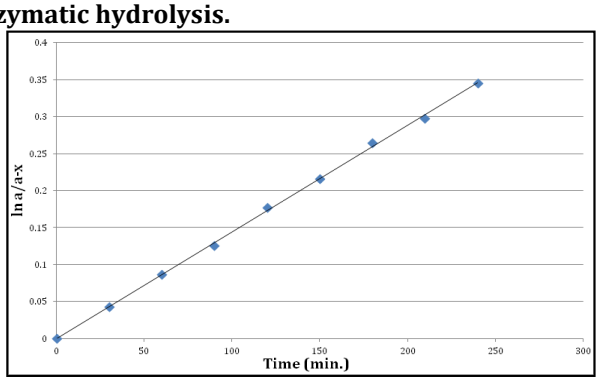


Table 3. Data obtained from kinetic study.

Absorbance	Medium	Time (min.)	x ($M\times10^6$)	a-x ($M\times10^6$)	ln a/a-x
0.1413	pH 1.2	30	0.9465	99.0535	0.0095
0.1476	pH 7.4		1.6522	98.3478	0.0167
0.1343	Serum		4.2282	95.7718	0.0432
0.1399	pH 1.2	60	1.9223	98.0777	0.0194
0.1452	pH 7.4		3.2694	96.7306	0.0332
0.1286	Serum		8.2776	91.7224	0.0864
0.1390	pH 1.2	90	2.5245	97.4755	0.0256
0.1425	pH 7.4		5.0633	94.9367	0.0520
0.1236	Serum		11.8402	88.1598	0.1260
0.1371	pH 1.2	120	3.8569	96.1431	0.0393
0.1406	pH 7.4		6.3291	93.6709	0.0654
0.1174	Serum		16.2625	83.7375	0.1775
0.1360	pH 1.2	150	4.6166	95.3834	0.0473
0.1382	pH 7.4		7.9274	92.0726	0.0826
0.1130	Serum		19.4265	80.5735	0.2160
0.1343	pH 1.2	180	5.8205	94.1795	0.0599
0.1361	pH 7.4		9.3271	90.6729	0.0979
0.1076	Serum		23.2524	76.7475	0.2646
0.1338	pH 1.2	210	6.1711	93.8289	0.0637
0.1333	pH 7.4		11.1925	88.8075	0.1187
0.1041	Serum		25.7489	74.2511	0.2977
0.1322	pH 1.2	240	7.3261	92.6739	0.0761
0.1314	pH 7.4		12.4500	87.5500	0.1330
0.0992	Serum		29.2212	70.7788	0.3456

a = conc. of prodrug at zero time; (a-x) = conc. of prodrug remaining for any time.

Table 4. Parameters obtained from kinetic study.

pH 1.2	pH 7.4	Serum
ε = 713	ε = 750.5	ε = 701
λ_{max} = 336 nm	λ_{max} = 354 nm	λ_{max} = 349 nm
$t_{1/2}$ = 2186.12 min	$t_{1/2}$ = 1250.90 min	$t_{1/2}$ = 481.25 min
k_{obs} = 0.000317 min^{-1}	k_{obs} = 0.000554 min^{-1}	k_{obs} = 0.001440 min^{-1}

ε = absorbance coefficient and K_{obs} = observed rate constants of hydrolysis.

CONCLUSION

The synthetic plan was designed to use the principle of coumarin-based prodrug system to synthesize a prodrug for treatment of NSCLC that can deliver three active moieties: 5-FU, LV and 5,7-dimethoxy-4-phenylcoumarin. Based on *in-vitro* kinetic studies, the synthesized prodrug showed a significant stability in buffers of physiological pH values and hydrolyzed enzymatically in human serum followed pseudo order kinetics. Consequently, it is believed that the synthesized prodrug may be a potential candidate as oral prodrug for treatment of NSCLC, and is a first agent belongs to a new prodrug strategy, which is a coumarin-based triple mutual prodrug.

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