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Mutual Prodrug of 5-Ethynyluracil and 5-Fluorouracil: Synthesis and Pharmacokinetic Profile

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Abstract

The oral administration of the standard cytotoxic agent 5-fluorouracil is extensively limited in the last three decades. This restriction is due to the drug's uneven intestinal absorption due to the changeable activity of dihydropyrimidine dehydrogenase, an enzyme found in the intestinal mucosa. A prodrug containing 5-fluorouracil and 5-ethynyluracil was developed in this study to allow mutual release of these two active medicines *via* a lactonization-facilitated release mechanism. Using coumarin as a precursor, the synthesis of the target prodrug was carried out in seven stages. The chemical backbones of the synthesized intermediate molecules and the target prodrug were verified using spectra acquired from several spectrophotometers, including FTIR, 1H-NMR, and 13C-NMR. In the HCl- (pH 1.2) and phosphate- (pH 6.8) buffers, the target prodrug's chemical stability was studied. Human serum was also used to test the prodrug's ability to release its active components. Chemical stability tests revealed that the targeted prodrug had significant stability in the HCl-buffer, with a t1/2 of 33.18 hours, and in the phosphate-buffered saline, with a t1/2 of 18.14 hours, according to pseudo-first-order kinetics. Furthermore, the prodrug may liberate the two active compounds in human serum with a t1/2 of 4.62 hours using zero-order kinetics. The authors came to the conclusion that the target prodrug might be a good mutual prodrug for oral ingestion of 5-fluorouracil and 5-ethynyluracil.

Keywords: Cyclization •Pharmacokinetic •Chemical •Kinetics

Introduction

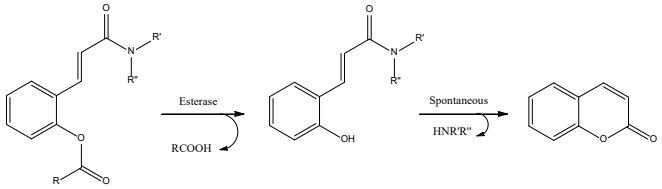
5-Fluorouracil (FU) has been used to treat a variety of cancer morphologies since it was first designed and synthesized in 1957 [1]. Nonetheless, due to its high frequency of adverse effects, poorer target ability, and low tumor sensitivity due to established resistance, the chemotherapeutic use of this tumor-fighting agent is being avoided [2,3]. Several advancements have been studied to control these hurdles, including adjusting administration programs, adjusting metabolic fates, creating and synthesizing novel fluoro-pyrimidines, and using diverse prodrug tactics [4-7].

Inside the viable cell, FU must be transformed *via* different metabolic pathways and steps into active forms since it is a prodrug [8]. The enzyme named dihydropyrimidine dehydrogenase and housed in the liver (DPDE) accounts for the basis of extracellular destruction of the plurality of the FU dose [9].

The utility of FU as an oral cytotoxic agent has been questioned because of its unpredictable GIT absorption that subsequently results in the fluctuation of the plasma levels with significant intra- and inter-individual versions. These outcomes could attribute to the changeable potential of DPDE localized in the GIT mucosa [10,11].

To enhance the oral bioavailability of the FU chemotherapy, interfering with the negative role of DPDE through its inhibition has become a potential target [12]. Although there are many evaluated inhibitory compounds, those analogs to FU exhibited the highest potential as DPDE in activators and 5-Ethynyluracil (EU) was the best [7,13-18].

Through the past 50 years, much interest has polarized to the design and synthesis of the Lactonization-Facilitated Release (LFR) prodrugs[19-23]. This prodrug phenotype is valuable to enhance the therapeutic potency of many polar drugs by modulating their hydrophilicity or shifting their metabolism to another direction [24,25]. The LFR prodrug system, as depicted in reveals many benefits, such as the eloquent release of the active component (s) when the LFR prodrug assaults *via* esterase enzyme Scheme 1. Besides, the rate of releasing the active component (s) may be modified through the presence of various functional groups on the coumarin chemical nucleus [26,27]. Moreover, the last compound, coumarin, acquired since the active component (s) is free from the LFR prodrug is documented to be safe [28].



Scheme1. The activation two steps of the LFR prodrug system.

The goal of this paper is to use the LFR prodrug system to develop and synthesize a mutual prodrug. This LFR prodrug can release FU as an oral cytotoxic agent and EU as a metabolic modulator when activated. To achieve this goal, the chemical stability of the synthesized prodrug was assessed, as well as its release patterns, using two buffer systems that simulated the GIT and human plasma, respectively.

Materials and Methods

Experimental

Chemicals and solvents were obtained from foreign sources for manufacturing the LFR prodrug and its intermediate components, as well as analyzing *in vitro* release. Shimadzu LCMS-2020 with an electrospray ionization source was used to scan the mass spectrum, Bruker Advance

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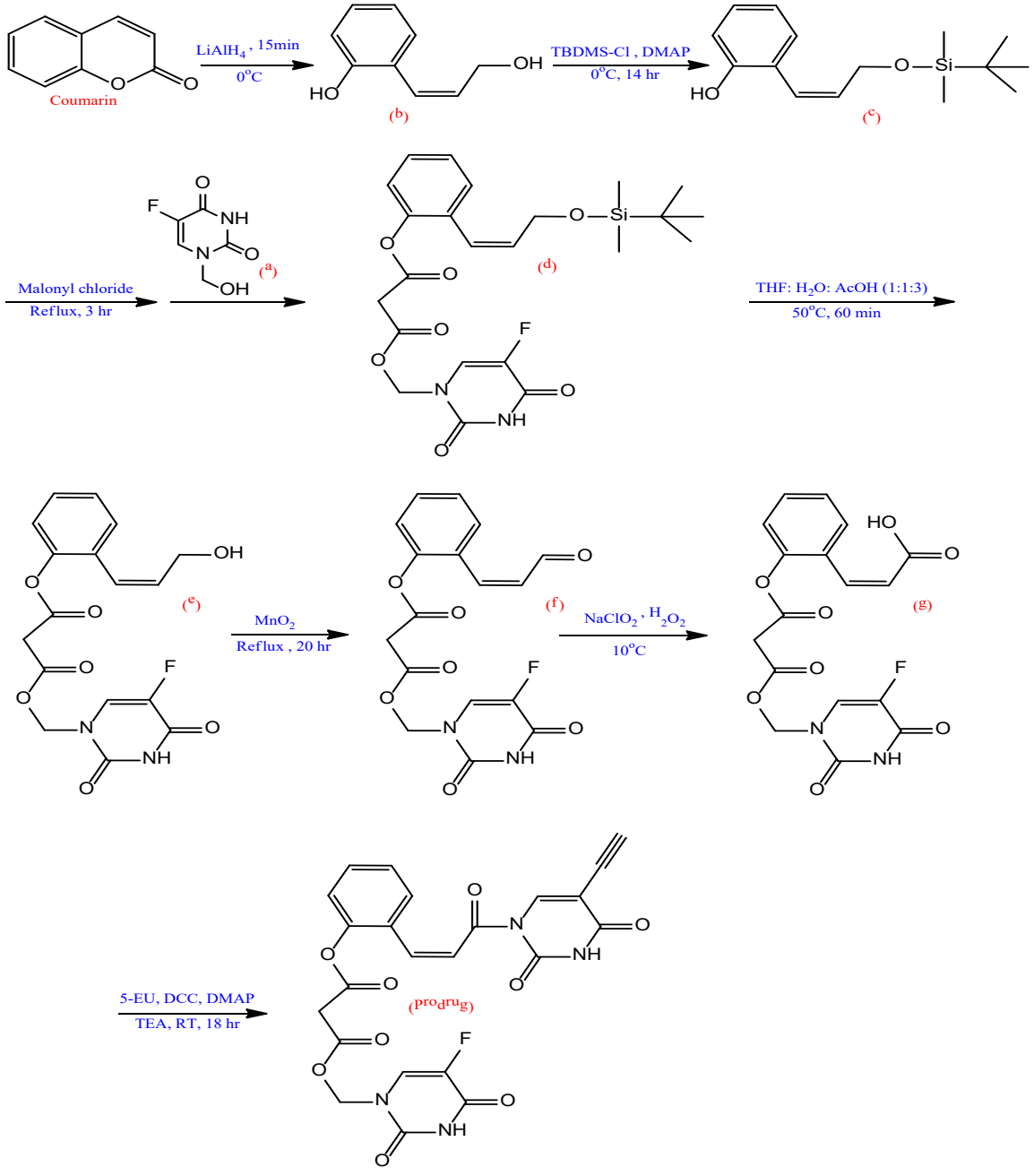
DRX-400 MHz was used to identify the NMR spectra, and Bruker-Alpha ATR-FTIR was used to screen the IR spectrum to validate the chemical structures of the synthesized products. Varian UV/Visible spectroscopy was used to determine the UV spectra of the LFR prodrug and its reaction intermediates. The *in vitro* stability and release experiments were also monitored using the same device. The course of the reactions and also purity of products were monitored using Thin-Layer Chromatography (TLC). The stationary and mobile phases were precoated silica gel plates and a chloroform: Acetone (4:1) eluent system, respectively.

Chemical synthesis

The LFR prodrug was synthesized using the synthetic approach shown



Scheme2. Synthetic pathway of the LFR prodrug.

in Scheme 2.

Synthesis of the intermediate a: At 60°C, a solution was produced by magnetically agitating a combination of formaldehyde (5 ml, 37%) and FU (1.04 g, 8 mmol) in 25 ml H2O for 45 minutes. Under decreased pressure, the resulting solution was evaporated to dryness, and the named product was recrystallized from EtOH [29,30].

Synthesis of the intermediate b: A solution of coumarin (25 mmol, 3.65 g) in 50 ml dry ether was mixed with a solution of lithium aluminum hydride (50 mmol, 1.9 g, $LiAlH_4$) in 50 ml dry ether in an ice bath. The resulting mixture was agitated for 15 minutes before being treated with HCl (27 mL, 5%), yielding a pH 5 solution. The organic layer was dried over $MgSO_4$, filtered, and vaporized after the crude was extracted with ether (350 ml). From EtOH, the intermediate b was recrystallized [31].

Synthesis of the intermediate c: The solution of tert-butyldimethylsilyl chloride (25 mmol, 3.79 g, TBDMS-Cl) in 35 ml dry THF was treated in an ice bath with the solution of b (22.8 mmol, 3.43 g) in 40 ml dry THF. A solution of 4-dimethylaminopyridin (34 mmol, 4.18 g, DMAP) in 40 ml dry THF was drop wise added to the resulting combination. The reaction mixture was agitated for 14 hours, then filtered and evaporated until it was

completely dry. The crude was dissolved in EtOAc (50 ml) and washed with HCl (50 ml, 1 N), $NaHCO_3$ (25 ml, 5%), and H_2O in that order (25 ml). The organic layer was filtered and evaporated after being dehydrated with $MgSO_4$. From $CHCl_3$, the intermediate c was recrystallized [31,32].

Synthesis of the intermediate d: Malonyl chloride (10 mmol, 1 ml) was added to a combination of c (10 mmol, 2.65 g) and a (10 mmol, 1.60 g) in 50 ml dried $CHCl_3$. With continual stirring, the mixture was refluxed for 3 hours. TLC was used to monitor the progress of the reaction using a combination of EtOAc and ether as an eluent. The sample solution was dried over anhydrous $MgSO_4$, condensed under minimized pressure, and purified in column chromatography with a $CHCl_3$: EtOH (2:1) combination, yielding the named product [31,33].

Synthesis of the intermediate e: In a combination of H_2O (10 ml), THF (10 ml), and acetic acid, intermediate d (4 mmol, 1.97 g) was dissolved (30 ml). The mixture was vaporized under decreased pressure after 60 minutes of stirring at 50°C. The crude was dissolved in 50 mL EtOAc, and the resulting solution was washed with 5% $NaCO_3$ (50 mL, 5%) (50 ml). The organic layer was dehydrated with $MgSO_4$, filtered, and evaporated at low pressure. From EtOH, the intermediate e was recrystallized [34,35].

Synthesis of the intermediate f: MnO_2 (20 mmol, 1.74 g) and intermediate e (4 mmol, 1.51 g) were suspended in $CHCl_3$ (30 ml) and refluxed for 20 hours. The heated mixture was filtered, and the solid that emerged was washed in 30 ml warm $CHCl_3$. Under decreased pressure, the organic layer was evaporated, and the crude was dissolved in 30 ml propanone and filtered. The intended product was obtained once the solution was vaporized [36].

Synthesis of the intermediate g: An aqueous solution of the intermediate f (4 mmol, 1.50 g), NaH_2PO_4 (0.85 mmol, 102 mg), and H_2O_2 (30 percent, 4.17 mmol, 0.5 ml) in 25 ml ACN was progressively added. Using an ice-water bath, the temperature of the reaction was kept below 10°C during the addition procedure, and oxygen bubbles were seen in the reaction mixture. When the bubbles ceased forming, 0.05 g Na_2SO_3 was added to destroy the unreacted H_2O_2 and HOCl. The reaction mixture was acidified to pH 2 with 1N HCl before being extracted with 50 ml EtOAc. The organic layer was dried over $MgSO_4$, filtered, and vaporized after being washed with 25 ml brine. The crude was dissolved in ACN and filtered after being treated with an aqueous $NaHCO_3$ solution to achieve a pH of 6.5. As the filtrate was acidified to pH 3 with 1N HCl, the desired product was separated [37,38].

Synthesis of the LFR prodrug

5-EU (2 mmol, 0.27 g), DCC (2.4 mmol, 0.5 g), DMAP (0.17 mmol, 20 g), and TEA (2 mmol, 0.3 ml) were serially added to a solution of g (2 mmol, 0.78 g) in 50 ml freshly distilled DMSO put in an ice-water bath. For 18 hours, the mixture was mixed at room temperature. The reaction mixture was then treated with 5 ml MeOH and 0.5 ml acetic acid, agitated for 60 minutes, then neutralized with an aqueous $NaHCO_3$ solution. The crude was washed with H_2O after the precipitate was filtered, the filtrate was vaporized, and the filtrate was vaporized. A combination of EtOH and CHCl3 (2:1.5) was used to recrystallize the target compound [31].

In vitro kinetic studies

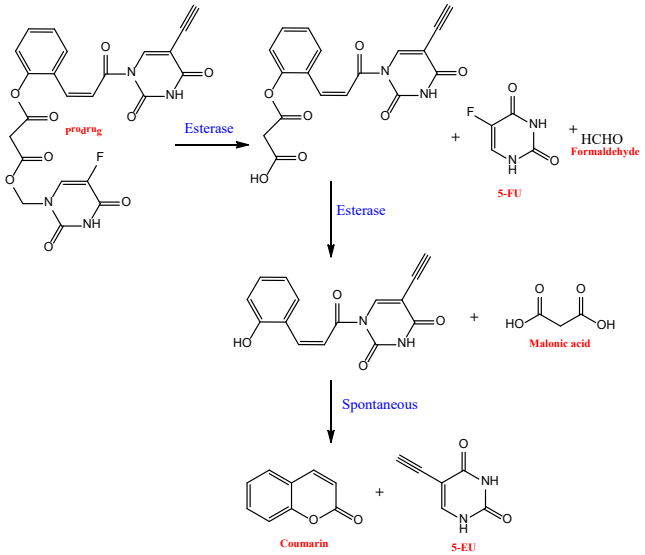
Chemical stability: The chemical stability of the produced prodrug was tested in two pH buffers: HCl (pH 1.2) buffer and phosphate-buffered saline buffer (pH 6.8) [39,40]. The following mathematical formula of Beer's law was used to track the reduction in prodrug concentration vs time in this investigation using UV/Visible spectroscopy: "Absorbance = $\varepsilon \times L \times C$" [41].

L is the path length of the cell holder (2 cm), C is the prodrug concentration, and ε is the absorbance coefficient.

A warmed LFR prodrug (5 mol) solution in 2 ml DMSO was combined with 48 ml prepared buffer solution. The timer was started, and the resulting solution was kept at 37°C in a warm water bath for preservation. The solution was then divided into a set of ten test tubes, each containing 5 ml. An individual test tube was chosen for each time interval of 0.5, 1, 1.5, 2, 2.5, 3, 3.5, or 4 hours, and its contents were mixed with 2 ml dichloromethane. To identify the remaining concentration of the prodrug, the aqueous aliquot (2 ml) was spectrophotometrically assessed at designated λmax [42,43].

Enzymatic hydrolysis

The hydrolysis of the produced LFR prodrug in human serum was monitored using an approach similar to that used to investigate chemical stability. The investigation was done by observing the rise in the concentration of 5-EU versus time, with the exceptions of replacing the buffer solution with serum and replacing the buffer solution with serum [44–47]. Since 5-EU is the last product released from the mutual prodrug under the action of the esterase enzyme, as illustrated in the concentration of this agent was examined (Scheme 3).



Scheme 3. The possible mechanism of releasing the active moieties in human serum from the LFR prodrug.

Results and Discussion

Design of the LFR prodrug

The synthetic LFR prodrug was created in order to improve the therapeutic utility of FU as an oral drug. Three concerns were addressed in order to achieve this goal. The first is to choose a prodrug that increases FU's lipophilicity, reduces its degradation by DPDE, and allows for mutual effect. The log P values for the FU, EU, and target prodrug were determined to be -0.90, -0.51, and 1.76, respectively, in this regard. This suggests that the target LFR prodrug has a higher lipophilicity than its predecessor medications, which might help increase FU oral bioavailability. Furthermore, releasing FU and EU at the same time may minimize the degradation of FU by DPDE, allowing for mutual activity. The research of prodrug stability in medium with pH values that mimic those observed in the gastrointestinal system is the second topic. The final is the capacity of the synthesized LFR prodrug to release FU and EU simultaneously in a human serum with an appropriate half-life.

Synthetic pathway

The synthesis of the LFR prodrug involves a seven-step linear process, as illustrated in Scheme-2, and is a simple variant on the one described by Mustafa and Al-Omari [31]. This version used a malonyl linkage to bind the phenolic hydroxyl group of the carrier molecule to the phenolic hydroxyl group of chemical a. $LiAlH_4$ was used to convert coumarin into an open ring diol under very strict circumstances. To avoid the reduction of the exocyclic double bond, the temperature was controlled below 0°C and the reaction duration was kept under 15 minutes, and the catalyst was of high purity to

reduce side reactions. TBDMS-Cl was used to protect the allylic hydroxyl group as silyl ether in the second phase. The phenolic hydroxyl group then collaborated with the previously synthesized chemical as to produce a diester connection utilizing malonyl chloride as an anchor in the following step. In the fourth stage, an acid deprotected the allylic hydroxyl group, which was then oxidized into allylic aldehyde by a selective oxidizing agent, MnO_2, in the next step. In the last synthesis stages, $NaClO_2$ and H_2O_2 were used to oxidize the allylic aldehyde to allylic carboxylic acid, which was then linked with EU through DCC to yield the target LFR prodrug.

Structural characterization

The physicochemical parameters and spectral data for the target LFR prodrug and its reaction intermediates obtained from the used instruments are provided in the supplementary file. The chemical frameworks of the produced compounds were validated by these findings.

In vitro kinetic studies

Chemical stability: The LFR prodrug displayed significant chemical stability in the HCl buffer and phosphate-buffered saline under experimental circumstances, with half-lives of 33.19 hours and 18.13 hours, respectively. This stability may be due to the steric hindrance surrounding the ester bonds, which provides excellent resistance to nucleophilic attack [48]. This discovery also indicated that the prodrug can pass through the media intact with a pH range similar to that of the gastrointestinal system [49–51].

Despite the fact that the hydrolysis of the LFR prodrug in the used buffers is dependent on two parameters, including the prodrug and attacking agent concentrations, the kinetics was observed to be pseudo-first-order [52–54]. This is because the attacking agent's concentration is exceedingly high in relation to the prodrug's, causing its influence on the hydrolysis kinetics to be overlooked [55].

Release study

The prepared prodrug was capable of releasing the active two moieties in a zero-order kinetics manner, with a t1/2 of 4.62 hours (Tables 1-3). This discovery demonstrated that the LFR prodrug had a long enough circulation time to reach the target and free the two drugs [56]. The LFR prodrug can be administered orally in a low-frequency mode, based on the kinetics characteristic [57], as a result of which patient compliance has improved [58].

Table1. Kinetic findings gathered from the chemical stability study in HCl (pH 1.2) buffered solution.

Absorbance	Time(hr)	X (M × 106)	a-x (M × 106)	In a/a-x
0.1328	0.0	0.0000	100.0000	0.0000
0.1316	0.5	0.9411	99.0589	0.0095
0.1303	1.0	1.8742	98.1258	0.0189
0.1288	1.5	3.0120	96.9880	0.0306
0.1282	2.0	3.4639	96.5361	0.0353
0.1267	2.5	4.6256	95.3744	0.0474
0.1258	3.0	5.2711	94.7289	0.0542
0.1240	3.5	6.6265	93.3735	0.0686
0.1231	4.0	7.2786	92.7214	0.0756
Note. a= prodrug concentration at zero time that equals to 100 M, and (a-x)=Residual concentration of prodrug at defined time.				

Table 2. Kinetic findings gathered from the chemical stability study in phosphate-buffered solution (pH 6.8)..

Absorbance	Time(hr)	X (M × 106)	a-x (M × 106)	In a/a-x
0.1301	0.0	0.0000	100.0000	0.0000
0.1280	0.5	1.6329	98.3671	0.0165
0.1259	1.0	3.2414	96.7586	0.0330
0.1240	1.5	4.6887	95.3113	0.0480
0.1215	2.0	6.6103	93.3897	0.0684
0.1198	2.5	7.9020	92.0980	0.0823
0.1177	3.0	9.5311	90.4689	0.1002
0.1162	3.5	10.6841	89.3159	0.1130
0.1140	4.0	12.3422	87.6578	0.1317
0.1267	4.0	44.6184		

Table 3. Kinetic findings gathered from the *in vitro* release study of the LFR prodrug in human serum.

Absorbance	Time (hr)	x (M × 106)
0.0000	0.0	00.0000
0.0954	0.5	5.8080
0.0989	1.0	11.7207
0.1032	1.5	17.4477
0.1079	2.0	22.4918
0.1118	2.5	27.9017
0.1167	3.0	33.4394
0.1216	3.5	38.9771

Provide the results of the *in vitro* kinetic investigations, whereas Table 4 shows the calculated kinetic parameters depicted a graph illustrating the association between the released concentration of EU and the passage of time (Figure 1).

Table 4. Findings gathered from the kinetic studies.

HCl (pH 1.2) buffer	Phosphate-buffered saline (pH 6.8)	Serum
ε= 284 L mol^{-1} cm^{-1}	ε=298 L $mo1^{-1}$ cm^{-1}	ε=1930.86 L $mo1^{-1}$
λ_{max}=281 nm	λ max=312 mm	λ max =292 nm

$t_{½}$ =33.19 hr	$t_{½}$=18.13 hr	$t_{½}$=4.62 hr
k_{oba}=5.8 × 106 hr^{-1}	k_{oba}=10.62 × 106 hr^{-1}	k_{oba}=10.83 × 106 $M.hr^{-1}$
ε=Absorbance coefficient, and Koba=observed rate constant.		

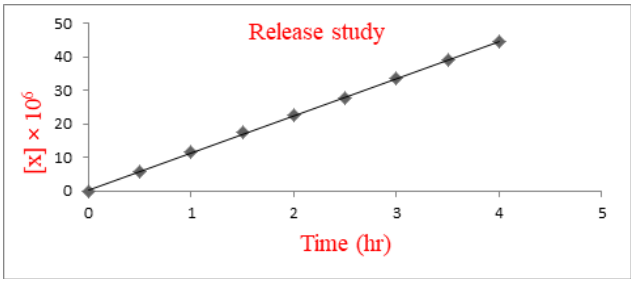


Figure 1. Graphical illustration of the association between the released concentration of EU and the time.

Conclusion

Using an LFR prodrug system, this research found that FU and its powerful metabolic modulator, EU, may be combined into a single chemical entity. The synthesized mutual prodrug was stable in medium with pH values that mimicked those observed in the gastrointestinal system, according to *in vitro* kinetic tests. In addition, the LFR prodrug was able to release FU and EU in a human serum with a t1/2 of 4.62 hours, following zero-order kinetics. The t1/2 value enables the LFR prodrug to reach the target intact, resulting in an increase in therapeutic effectiveness.

Conflict of interest

The authors stated that there is no conflict of interest.

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