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Conjugation of Sinapic Acid Analogues with 5-Fluorouracil: Synthesis, Preliminary Cytotoxicity, and Release Study

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ABSTRACT
Tripartite prodrug approach is a promising strategy to improve the therapeutic efficacy of orally administrated drugs having a low bioavailability. 5-Fluorouracil is a primary chemotherapeutic agent used in the treatment of many solid tumors. The oral use of 5-fluorouracil suffers from many challenges, which are principally contributed to the erratic activity of dihydropyrimidine dehydrogenase in the GIT. In this work, five integrates were prepared as tripartite mutual prodrugs by connecting sinapic acid and four of its analogues to 5-fluorouracil through amenable ester bond. Chemical structures of the prepared integrates were defined by studying their FTIR, ^{1}H-NMR, and ^{13}C-NMR spectra. Initiatory cytotoxicity study was verified for these integrates via MTT test versus four cancer cell lines including HeLa, MCF-7, AMN3, and SKG. The in vitro study of releasing the active drug from the synthesized integrates was followed spectrometerically using a human serum. The results of the cytotoxicity study after 24 hr of treatment revealed that the synthesized integrates by themselves have a non-toxic activity toward the test cell lines. In contrast, the results of the same study but after 72 hr of treatment indicated the potential antitumor activity of the tested prodrugs which indicates the effective release of the active components from these prodrugs. Also, the data gathered from the release study utilizing a human serum indicated that these integrates can release the active agents pursued pseudo first order kinetics. It is concluded that the synthesized integrates could be considered as mutual prodrugs and that may be improved the clinical applications of 5-Fluorouracil.

Key words: 5- Fluorouracil, Sinapic acid, Mutual prodrug, Antitumor, Release study, Kinetics.

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INTRODUCTION
5-Fluorouracil (5-FU), a traditional antimetabolite, exerts its cytotoxic effect via the inhibition of cellular thymidylate synthase and mismerging into DNA and/or RNA to disturb the vital roles of these nucleic acids. As a single agent or a component of different regimens, 5-FU has been applied since its invention in 1957 in treatment of various types of cancer such as colorectal, stomach, pancreatic, head, and breast cancers (1).

However, there are several drawbacks which limit the clinical applications of 5-FU such as its toxic effects, short plasma half-life which is principally because of the dihydropyrimidine dehydrogenase, atypical absorption with variable plasma levels following oral administration, and sensitivity to resistant mechanisms developed by the cancer cells (2).

To transact with these issues, great efforts have been developed and assessed over the years. One of the most applicable attempts is the incorporation of 5-FU into a different types of prodrugs, which are designed to either target the tumor cells or avoid certain degradation metabolic pathways (3,4).

Prodrug strategy is still recognized as a productive area of research in medicinal chemistry. Conceptually, the prodrug is an inert moiety that can be regenerated the active substance upon degradation. For the carrier-linked prodrug, the carrier is a non-toxic molecule which selected according to certain criteria (5). The mutual prodrug is designed where the carrier is a biologically active molecule which can improve the pharmacological or the physicochemical properties of the active agent without a negative interference (6).

Phenolic compounds are naturally occurring secondary metabolites found majorly in a plant kingdom and these metabolites exhibit a wide range of pharmacological properties which make them an important class of phytochemicals (7). The definitive group of this important class is known as a hydroxycinnamic acids family, to which caffeic, vanillic, ferulic, para-coumaric, and sinapic acids are belonged (8).

Sinapic acid (SA) is commonly detected in the usual human diet because of its predominance in many herbs, cereal grains, fruits, and vegetables (9). Many research papers demonstrated the in vitro and in vivo biological properties of SA reported that it has many interesting pharmacological properties such as antidiabetic, anxiolytic, analgesic, antibacterial, antioxidant, and anti-inflammatory effects (10–17). Concerning its antitumor activity, SA exhibits dose- and time-dependent effect on some investigated cancer cell lines (18). Since now, the existing information found in the literature about the antitumor activity of SA is limited (19).

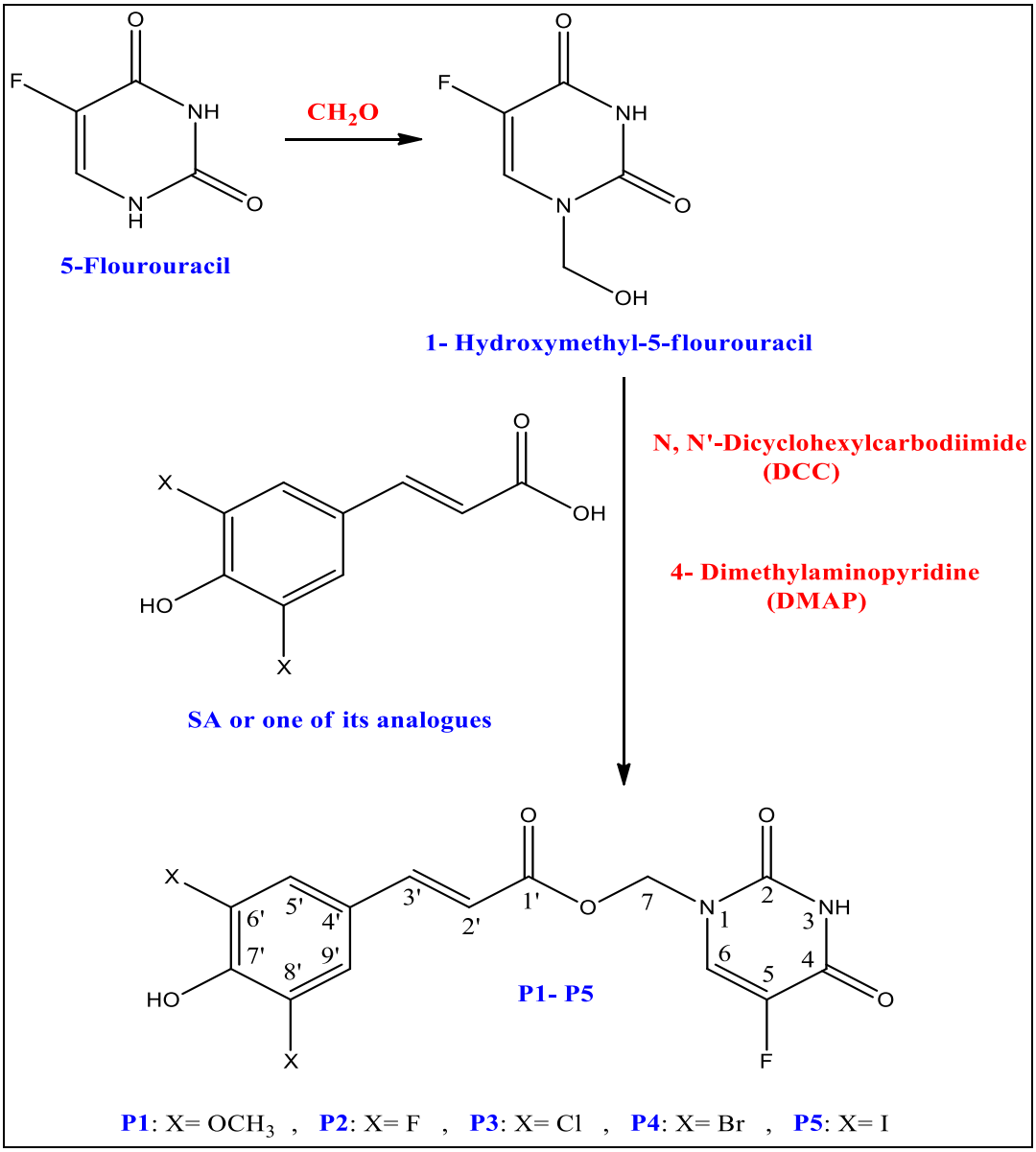
The aim of this work is to apply the tripartite mutual prodrug approach for improving the clinical utilization of 5-FU. This can be achieved by grafting this cytotoxic drug to a series of SA and its four analogues via an ester linkage, examining the initiatory antitumor activity of the synthesized prodrugs, and finally following their in vitro hydrolysis spectroscopically to detect the efficiency of their release in a human serum.

MATERIAL AND METHODS
Chemicals and reagents utilized in this study were acquired from Sigma-Aldrich, Bio-World, and Tokyo Chemical

Industry. TLC investigations were followed on silica gel Si 60 F_{254} plates provided via Merck using a mobile phase consists of $CHCl_3$: acetone (4:1). Electrochemical CIA 9300 apparatus was employed to detect the melting points of synthesized tripartite mutual prodrugs and they were corrected. FTIR spectra of these prodrugs were examined on Bruker-Alpha ATR spectroscopy while their mass spectra expressed as a mass-to-charge (m/z) ratio were determined via Shimadzu LCMS-2025 spectrometer, which was adjusted in the positive mode utilizing electrospray ionization (ESI) technique. Varian UV/ Visible spectrometer was employed to identify the wavelength of maximum absorption and to monitor the release study. ^{13}C-NMR (125 MHz) as well as ^{1}H-NMR (500 MHz) spectra of these prodrugs were screened on Bruker 500 MHz AVANCE III HD NMR Spectrometer using TMS as an internal reference.

Chemical synthesis

The plan followed in the chemical synthesis of tripartite mutual prodrugs (P1-P5) is observed in Scheme 1.



Scheme 1: The plan followed in the synthesis of tripartite mutual prodrugs (P1-P5) .

Synthesis of SA and its analogues

SA and its analogues were prepared according to *Mustafa* (2019) without important alterations. The selected analogues have one of the following substitutions: F, Cl, Br, or I (19).

General method for the synthesis of tripartite mutual prodrugs (P1-P5)

The suspension of 5-FU (1.04 g, 8 mmol) in an aqueous solution of formaldehyde (5 ml, 37%) was stirred until the solution produced (1 hr). Subsequently, the resultant mixture was stirred in an ice-bath. As the temperature drops to 0°C, the following additions were carried out: dry

acetonitrile (12 ml), SA or one of its analogues (11.2 mmol), DCC (2.30 g, 11.2 mmol) and DMAP (0.064 g, 0.52 mmol) and the reaction mixture stirred at that temperature for 1.5 hr., and at room temperature for 12 hr. The formed dicyclohexylurea (DCU) was eliminated via filtration, then the solvent was concentrated under reduced pressure. The residue was dissolved in ethyl acetate (50 ml), washed with 1N HCl (10 ml) then 10% $NaHCO_3$ (20 ml) followed by saturated NaCl aqueous solution (25 ml). The organic layer was dried over dry $MgSO_4$ and evaporated to afford the crude product, which was recrystallized from methanol (20).

Physical attributes and structural characterization of tripartite mutual prodrugs (P1-P5)

(*E*)-N1- methyl-5-flourouracil-yl-1'-(6',8'-dimethoxy-7'-hydroxy) cinnamate (P1): White powder; 69% Yield; mp 109-111°C; UV (EtOH) λ_{max} 309; R_f 0.62; IR ν_{max} 3336, 3153, 3115, 3040, 2926, 1726, 1660, 1648, 1608, 1542, 1321, 1230, 1028 cm^{-1}; ^{1}H-NMR (DMSO-d_6, 500 MHz): δ= 11.80 (1H, s, N-3), 8.62 (2H, s, H-5', H-9'), 8.02 (1H, d, *J*= 15 Hz, H-3'), 7.84 (1H, s, H-6), 6.56 (1H, s, OH-7'), 6.42 (1H, d, *J*= 15 Hz, H-2'), 5.88 (2H, s, H-7) ppm; ^{13}C-NMR (DMSO-d_6, 125 MHz): δ= 162.7 (C, C-1'), 161.2 (C, C-4), 156.12 (C, C-2), 150.2 (C, C-7'), 145.4 (CH, C-3'), 136.6 (C, C-6', C-8'), 135.0 (CH, C-5', C-9'), 133.2 (C, C-5), 130.5 (C, C-4'), 119.1 (CH, C-2'), 110.5 (CH, C-6), 75.1 (CH_2, C-7) ppm; MS-ESI spectrum (m/z): 367 $[M+H]^+$.

(*E*)-N1-methyl-5-flourouracil-yl-1'-(6',8'-diflouro-7'-hydroxy) cinnamate (P2): White powder; 64% Yield; mp 96-99°C; UV (EtOH) λ_{max} 302; R_f 0.50; IR ν_{max} 3340, 3150, 3110, 3043, 2906, 1723, 1661, 1647, 1612, 1539, 1230 cm^{-1}; ^{1}H-NMR (DMSO-d_6, 500 MHz): δ= 11.76 (1H, s, N-3), 7.73 (1H, s, H-6), 7.84 (1H, d, *J*= 15 Hz, H-3'), 6.80 (1H, s, H-8), 6.70 (2H, s, H-5', H-9'), 6.46 (1H, s, OH-7'), 6.38 (1H, d, *J*= 15 Hz, H-2'), 5.74 (2H, s, H-7) ppm; ^{13}C-NMR (DMSO-d_6, 125 MHz): δ= 166.3 (C, C-1'), 163.4 (C, C-4), 158.2 (C, C-2), 154.2 (C, C-6', C-8'), 144.6 (CH, C-3'), 134.8 (C, C-7'), 132.4 (C, C-5), 130.0 (C, C-4'), 119.9 (CH, C-2'), 115.4 (CH, C-5', C-9'), 110.8 (CH, C-6), 74.5 (CH_2, C-7) ppm; MS-ESI spectrum (m/z): 343 $[M+H]^+$.

(*E*)-N1-methyl-5-flourouracil-yl-1'-(6',8'-dichloro-7'-hydroxy) cinnamate (P3): White powder; 67% Yield; mp 107-110°C; UV (EtOH) λ_{max} 300; R_f 0.54; IR ν_{max} 3332, 3152, 3113, 3050, 2894, 1728, 1671, 1656, 1624, 1542, 1092 cm^{-1}; ^{1}H-NMR (DMSO-d_6, 500 MHz): δ= 11.64 (1H, s, N-3), 7.92 (1H, s, H-6), 7.82 (1H, d, *J*= 15 Hz, H-3'), 7.09 (2H, s, H-5', H-9'), 6.53 (1H, s, OH-7'), 6.28 (1H, d, *J*= 15 Hz, H-2'), 5.93 (2H, s, H-7) ppm; ^{13}C-NMR (DMSO-d_6, 125 MHz): δ= 167.4 (C, C-1'), 164,4 (C, C-4), 159.2 (C, C-2), 155.8 (C, C-7'), 146.2 (CH, C-3'), 137.5 (C, C-5), 132.5 (CH, C-5', C-9'), 130.9 (C, C-4'), 122.8 (C, C-6', C-8'), 117.2 (CH, C-2'), 112.4 (CH, C-6), 78.7 (CH_2, C-7) ppm; MS-ESI spectrum (m/z): 376 $[M+H]^+$.

(*E*)-N1-methyl-5-flourouracil-yl-1'-(6',8'-dibromo-7'-hydroxy) cinnamate (P4): White powder; 65% Yield; mp 116-119°C; UV (EtOH) λ_{max} 301; R_f 0.58; IR ν_{max} 3342, 3150, 3118, 3051, 2890, 1728, 1670, 1648, 1615, 1538, 1050 cm^{-1}; ^{1}H-NMR (DMSO-d_6, 500 MHz): δ= 11.90 (1H, s, N-3), 7.92 (H, s, H-6), 7.78 (1H, d, *J*= 15 Hz, H-3'), 7.31 (2H, s, H-5', H-9'), 6.44 (1H, s, OH-7'), 6.30 (1H, d, *J*= 15 Hz, H-2'), 5.60 (2H, s, H-7) ppm; ^{13}C-NMR (DMSO-d_6, 125 MHz): δ= 166.6 (C, C-1'), 164.2 (C, C-4), 162.7 (C, C-2), 160.2 (C, C-7'), 145.1 (CH, C-3'), 137.2 (CH, C-5', C-9'), 133.2 (C, C-5), 130.1 (C, C-4'), 117.9 (CH, C-2'), 114.1 (C, C-6', C-8'), 109.5 (CH, C-6), 73.8 (CH_2, C-7) ppm; MS-ESI spectrum (m/z): 465 $[M+H]^+$.

(*E*)-N1-methyl-5-flourouracil-yl-1'-(6',8'-diiodo-7'-hydroxy) cinnamate (P5): White powder; 58% Yield; mp 136-139°C; UV (EtOH) λ_{max} 302; R_f 0.60; IR ν_{max} 3331, 3155, 3112, 3049, 2912, 1727, 1669, 1650, 1616, 1546, 1039 cm^{-1}; ^{1}H-NMR (DMSO-d_6, 500 MHz): δ= 11.69 (1H, s, N-3), 7.94 (1H, s,H-6), 7.80 (1H, d, *J*= 15 Hz, H-3'), 7.59 (2H, s, H-5', H-9'), 6.42 (1H, s, OH-7'), 6.32 (1H, d, *J*= 15 Hz, H-2'), 5.75 (2H, s, H-7) ppm; ^{13}C-NMR (DMSO-d_6, 125 MHz): δ= 178.4 (C, C-7'), 170. 2 (C, C-4), 168.0 (C, C-2), 166.9 (C, C-1'), 145.8 (CH, C-3'), 141.0 (CH, C-5', C-9'), 136.4 (C, C-5), 132.8 (C, C-4'), 118.6 (CH, C-2'), 108.7 (CH, C-6), 86.3 (C, C-6', C-8'), 72.3 (CH_2, C-7) ppm; MS-ESI spectrum (m/z): 559 $[M+H]^+$.

Initiatory cytotoxicity study

In a plate of 96 wells, the specific cancer cell line was distributed to attained 10^4 cells for each well. The wells were treated after the detected period individually with a specific concentration of the tested integrates. The utilized concentrations (400, 200, 100, 50, 25, 12.5, 6.25 µg/ml) were prepared from a stock solution of 1mM in DMSO. The assay was carried out in twice trials (in the next 24 hr and in the next 72 hr of treatment) by ejecting the growth medium, positioning the MTT solution (29 µl, 3.32 mM), and then the treated cells were incubated at 37 °C for 1.5 hr. The absorbances of the untreated well (A_U) and treated well (A_T) were verified by a microplate reader operated at 492 nm. The following numerical equation was used to calculate the growth inhibition percent (GI %) (17):

$GI\ \% = (A_U - A_T)/A_U \times 100$

A_U and A_T represent the absorbances of untreated and treated wells, respectively.

Release study

The enzymatic hydrolysis of the prepared prodrugs was followed in vitro using a human serum. The kinetics of this hydrolysis was specified by monitoring the increase in the concentration of 5-FU versus time and calculated by utilizing a Beer-Lambert hybride formula, which is:

$A = \varepsilon \times L \times C$

The abbreviations A, ε, L, and C refer to the absorbance, molar extinction coefficient or called absorbance coefficient, path length of the cell holder (1cm), and concentration, respectively.

Briefly, the sample of the prodrug (2.5 µmol) was dissolved in a phosphate buffered saline (9.8 ml). Preheated serum (10 ml) was added with gentle stirring to the prior solution affording the final concentration of 100 µM. The mixture was divided into a series of 10 test tubes, each one of 2 ml, and they incubated in a water-bath adjusted at 37 ±1°C. At this point, the time was begun to follow. At elected time

periods of 30, 60, 90, 120, 150, 180, 210, 240 and 270 min, the content of a labeled test tube was extracted by CH_2Cl_2 (2 ml). Aliquot (1 ml) was taken from the aqueous layer and examined via UV/Visible spectrometer at λ_{max} = 272 nm to identify the growing concentration of the released 5-FU (21).

RESULTS AND DISCUSSION

Chemical synthesis

In the synthesis of tripartite mutual prodrugs, the coupling of 1-hydroxymethyl-5-flourouracil with SA or one of its analogues was executed with little competition (22). This is because the hydroxyl group of 1-hydroxymethyl-5-flourouracil has a better chance to nucleophilic attack the carbonyl carbon of SA (or one of its analogues) in the term of magnitude (as in the synthesis of P2-P5) or steric hindrance (as in the synthesis of P1) than the hydroxyl group of SA and its analogues. So, the formation of ester from this reaction is a more prominent than the generation of the dimer (23,24).

Based on the spectroscopic identification data listed in Methods and meterials section, and also their comparison with those investigated in the literature, the chemical structures of the synthesized tripartite mutual prodrugs are displayed in Figure 1.

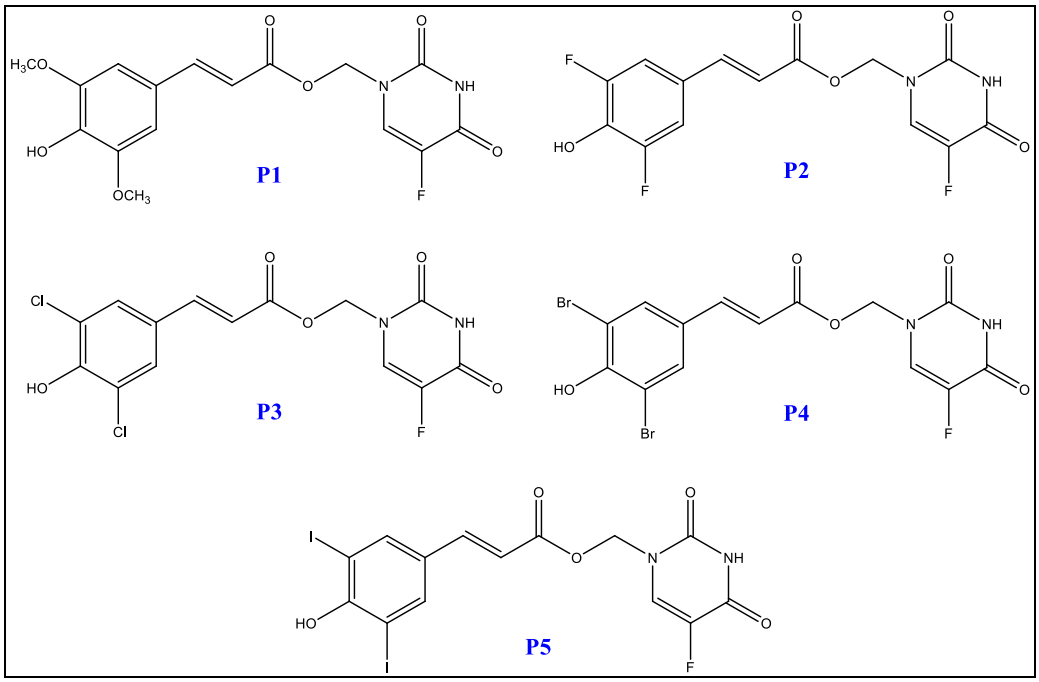


Figure 1: Chemical structures of the synthesized tripartite mutual prodrugs.

Initiatory antitumor activity

The synthesized tripartite mutual prodrugs were scanned for their initiatory antitumor activity against four cancer cell lines including HeLa (cervix), MCF-7 (breast), AMN3 (murine mammary adenocarcinoma), and SKG (esophageal). This assay was carried out by MTT test utilizing seven serial diluted concentrations of the tested prodrugs, DMSO as a negative standard, and 5-FU as a positive standard.

The results shown in Table 1 and pictured in Figure 2 revealed that the synthesized tripartite mutual prodrugs have considerable higher IC_{50} values calculated after 24 hr of treatment compared with that of a positive standard. This may be documented the assumption of the non-toxic nature of the prepared prodrugs (25,26).

The results found in Table 2 and characterized in Figure 3 indicated that the synthesized integrates may exhibit a higher antitumor activity aganist the test cell lines with an exceptional effect contributed to P2 compound . This activity which appeared after 72 hr of treatment may indicate that the synthesized prodrugs were hydrolyzed releasing 5-FU and SA (or one of its analogues) in the test medium. The IC_{50} values acquired after 72 hr of treatment were approximately lower than that of 5-FU, this may be indicated that the released products exerted an additional or synergestic antitumor activity to some extent against the test cell lines (21,27).

Table 1: Results gathered from examining the antitumor activity of the synthesized prodrugs after 24 hr of treatment.

Compound Name	HeLa	MCF-7	AMN3	SKG
5-FU	36.24 ± 1.20	34.47 ± 1.50	42.14 ± 1.10	43.16 ± 0.90
P1	249.22 ± 1.50	258.75 ± 1.05	289.10 ± 1.10	301.46 ± 1.20

P2	312.26 ± 1.45	304.46 ± 1.25	312.21 ± 1.50	309.89 ± 1.35
P3	327.12 ± 1.20	290.44 ± 1.35	298.67 ± 1.10	325.07 ± 1.25
P4	315.32 ± 1.15	294.37 ± 0.90	322.78 ± 1.30	327.12 ± 1.15
P5	318.29 ± 1.35	326.80 ± 1.00	326.14 ± 1.20	322.63 ± 1.20

The results expressed as IC_{50} ± SD, SD is detected for three independent experiments while IC_{50} is expressed in μM.

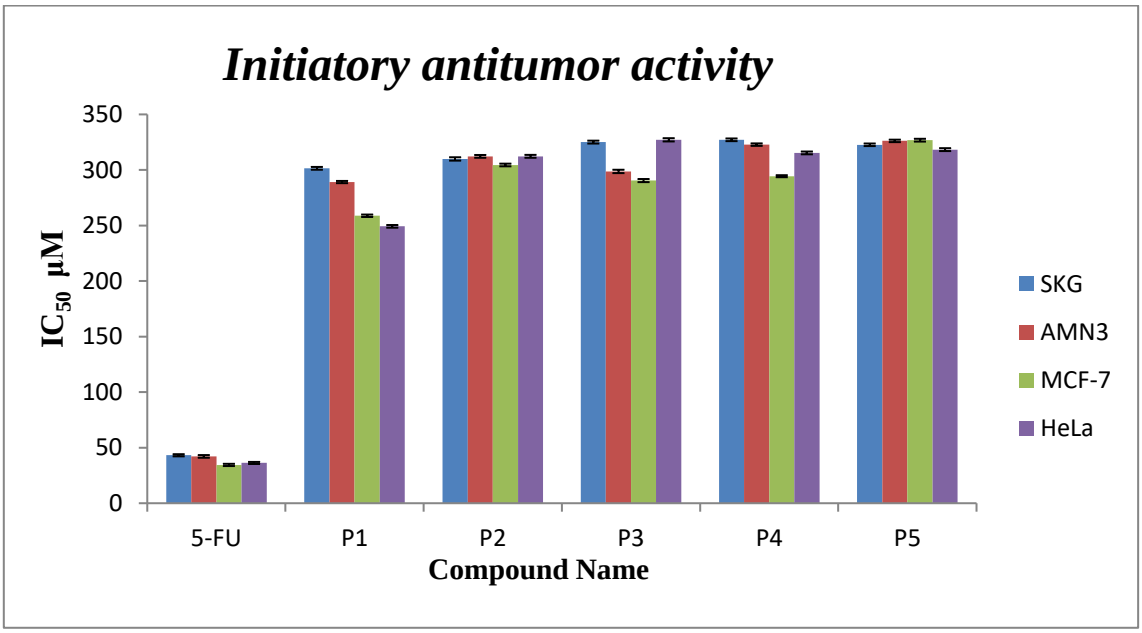


Figure 2: Diagram displayed the results acquired from initiatory antitumor activity, which was examined by MTT assay after 24 hr of treatment.

Table 2: Results gathered from examining the antitumor activity of the synthesized prodrugs after 72 hr of treatment.

Compound Name	HeLa	MCF-7	AMN3	SKG
5-FU	13.44 ± 0.92	12.86 ± 1.00	24.64 ± 1.20	22.17 ± 0.98
P1	11.36 ± 1.20	12.05 ± 1.10	19.64 ± 1.00	20.98 ± 1.25
P2	12.76 ± 1.50	12.08 ± 1.25	23.13 ± 1.20	22.47 ± 1.30
P3	13.47 ± 1.30	12.99 ± 1.50	22.78 ± 1.40	23.02 ± 1.45
P4	12.34 ± 1.20	12.30 ± 1.10	22.12 ± 0.90	23.34 ± 1.25
P5	13.49 ± 1.05	12.92 ± 1.15	25.02 ± 1.25	22.35 ± 1.30

The results expressed as IC_{50} ± SD, SD is detected for three independent experiments while IC_{50} is expressed in μM.

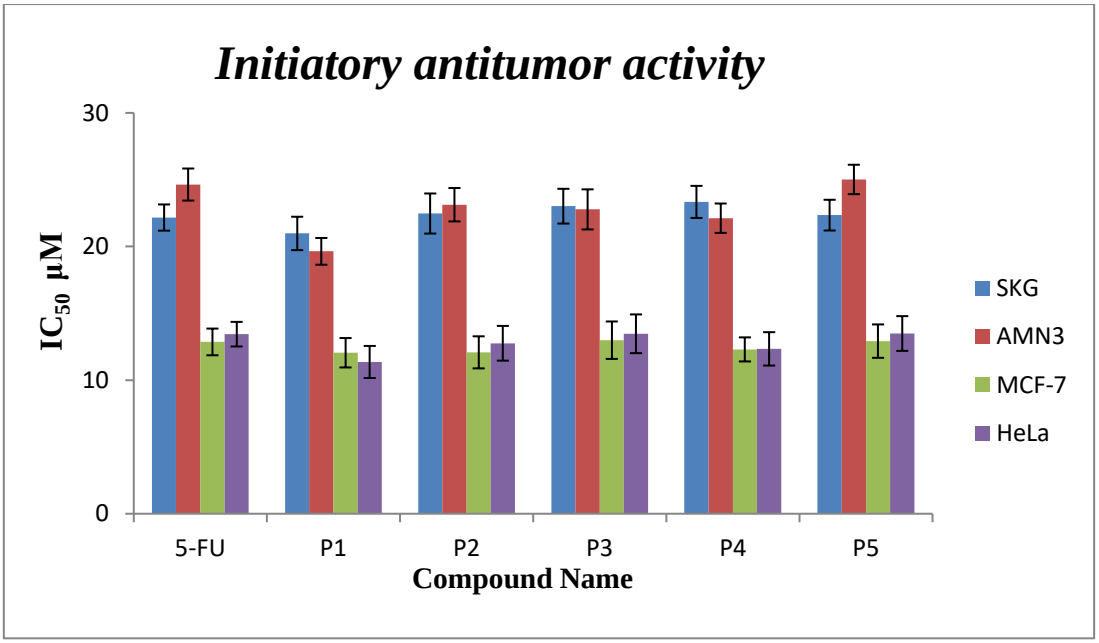


Figure 3: Diagram displayed the results acquired from initiatory antitumor activity, which was examined by MTT assay after 72 hr of treatment.

In vitro release study

Under the observational conditions, the synthesized prodrugs can liberate 5-FU, formaldehyde and SA (or one of its analogues) in a human serum adopted the pseudo first order kinetics with half-lives range 3-3.4 hr. This was confirmed by analyzing the kinetic parameters (Table 3, Figure 4) acquired from this study. The resultant half-lives were approximately within a small range, this may be due to the weak effect of the variation in the substitutions among the prepared prodrugs. This is because the different substitutes localized far from the attacking point, the carbonyl carbon of ester (28–30).

Table 3: Kinetic parameters acquired from the release study in a human serum.

Compound Name	ε	k_{obs} (min^{-1})	$t_{1/2}$ (min)
P1	261 ± 1.00	0.00369 ± 1.25	187.91 ± 0.90
P2	258 ± 1.30	0.00385 ± 1.15	180.12 ± 1.35
P3	264 ± 1.10	0.00350 ± 1.05	197.79 ± 1.22
P4	262 ± 1.25	0.00361 ± 1.15	191.82 ± 1.00
P5	267 ± 1.20	0.00373 ± 1.30	185.76 ± 1.10

ε is the absorbance coefficient and K_{obs} is the observed rate constants of hydrolysis. The results represented the mean ± SD of three separated experiments.

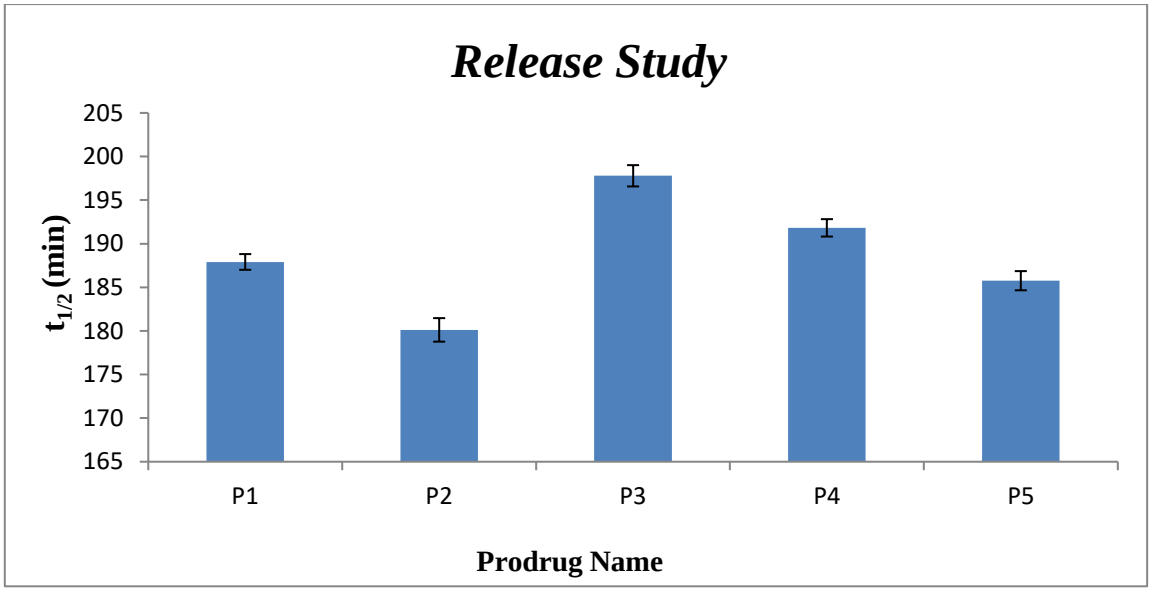


Figure 4: Diagram displayed the half-lives of the synthesized prodrugs (P1-P5).

CONCLUSIONS

This work highlighted the success in the synthesis of five tripartite mutual prodrugs by associating 5-FU to a series of SA and its analogues. From examining the antitumor activity next to 24 hr and 72 hr as well as the release study, it is deduced three concerning issues. The first reveals that the synthesized integrates have a poor antitumor activity versus the test cell lines after 24 hr of treatment, this is parallel to the assumption of regarding these integrates as prodrugs. The second issue is the synthesized integrates have a better antitumor activity than 5-FU after 72 hr of treatment, this indicates that the hydrolysis of these prodrugs by the cancer cells affording the release of the active components, which may be acted in an additional or synergistic manner. The third issue is the in vitro release study in a human serum was confirmed the second issue and also indicated the ability of these prodrugs to release the active components in relatively short half-lives. Accordingly, the synthesized tripartite mutual prodrugs may be considered as potential prodrugs for improving the clinical use of 5-FU.

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CONFLICT OF INTEREST

There are no conflicts of interest.

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