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Original article

Synthesis and biological activity evaluation of cytidine-5′-deoxy-5-fluoro-N-[(alkoxy/aryloxy)] carbonyl-cyclic 2′,3′-carbonates

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ABSTRACT

Capecitabine, an oral prodrug of 5-FU was developed to improve the tumor selectivity and tolerability. To enhance the efficacy of capacitabine, a series of 5′-deoxy-5-fluorocytidine derivatives **5a**–**e** were synthesized. In the present study, we investigated antitumor activity of 5′-deoxy-5-fluorocytidine derivatives both *in vivo* and *in vitro* methods. Title compounds were non-mutagenic to *Salmonella typhimurium* tester strain in Ames test. Compounds **5d** and **5e** are potent to inhibit the proliferation of NCI-69, PZ-HPV-7, MCF-7 and HeLa cells in MTT assay. In particular, **5d** and **5e** showed potent antitumor activities against L1210 leukemia cell line. Collectively, these findings suggest that **5d** and **5e** are more potent anti-cancer compounds than capecitabine.

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1. Introduction

Cancer is a common health problem worldwide, although cancer types vary considerably between affluent and less affluent countries [1]. Cancer is responsible for 1 in 8 deaths worldwide. In fact, cancer causes more deaths than AIDS, tuberculosis and malaria combined [2]. Cancer incidence and mortality rate are expected to rise steeply and will overtake heart disease as the number one killer of people around the world, according to a report by the International Agency for Research on Cancer (IARC) [2].

In spite of the use of surgical treatment and irradiation, chemotherapy still remains an important option for the treatment of solid cancers. Regardless of extensive research efforts, the therapeutic treatment of tumor patients is still not satisfying. Chemotherapeutic drugs are designed in such way that they preferentially target tumor cells without harming normal cells or tissues. Unfortunately, it often affects parts of the body not directly affected by the cancer itself in most cases, with harsh side effects due to the toxicity of the drugs. The indiscriminate nature of current cancer chemotherapy and drug resistance has initiated a search for more selective approaches.

Most antitumor agents are cytotoxic and are associated with severe side effects, including myleo-suppression, skin toxicity and intestinal toxicity. There have been several attempts to reduce such side effects by means of prodrug strategies for the tumor-selective delivery of cytotoxic agents [3]. The present study describes the tumor-selective delivery of 5-fluorouracil (5-FU) by sequential conversion of the new 5-fluoropyrimidine carbamate (**5a**–**e**) by endogenous enzymes preferentially localized in human liver and tumor tissue.

5-Fluorouracil (5-FU) is a pyrimidine analog and a standard antitumor agent acting as thymidylate synthase inhibitor belonging to antimetabolite family [4] and rapidly metabolized by enzyme dihydropyrimidine dehydrogenase (DPD) [5]. However, 5-FU is poorly tumor-selective, however, and its therapy causes highly incidences of toxicity in the bone marrow, central nervous system, gastrointestinal tract and skin. Thus, many compounds have been developed for advanced selectivity and to reduce toxicity such as UFT, 5-ethynyluracil, S-1(tegafur + 5-Chloro-2,4-dihydroxypyridine + Oxonic acid), tegafur, doxifluridine (5′-DFUR), carmofur and capecitabine [6]. Capecitabine

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(N4-pentyloxycarbonyl-5′-deoxy-5-fluorocytidine) is designed as a 'pro-drug' to the cytotoxic agent 5-FU and to be administered orally [7]. To improve the efficacy and safety profiles of the oral fluoropyrimidine 5′-DFUR, capecitabine, a new orally available 5-fluoropyrimidine carbamate, were synthesized so that it passes intact through the intestinal mucosa and then selectively delivers 5-FU to tumor tissues following its enzymatic conversion: first to 5′-deoxy-5-fluorocytidine (5′-DFCR) by cytidine deaminase, and finally to 5-FU by thymidine phosphorylase. The latter two enzymes are preferentially localized in tumor tissues. The catabolic pathway of 5-FU comprises dihydro-5-fluorouracil (FUH_2, via dihydropyrimidine dehydrogenase, DPD), 5-fluoro-ureidopropionic acid (FUPA) and fluoro-β-alanine (FBAL) [5]. To enhance the efficacy of capecitabine, but not its toxicity, we designed a series of 5′-deoxy-5-fluorocytidine derivatives, cytidine-5′-deoxy-5-fluoro-N-[(alkoxy/aryloxy)] carbonyl-cyclic 2′,3′-carbonates as prodrugs of known DPD inhibitor, 5-FU derivatives selectively in tumors, similar to the design of capecitabine [10]. These novel derivatives showed higher antitumor activity than capecitabine *in vivo*.

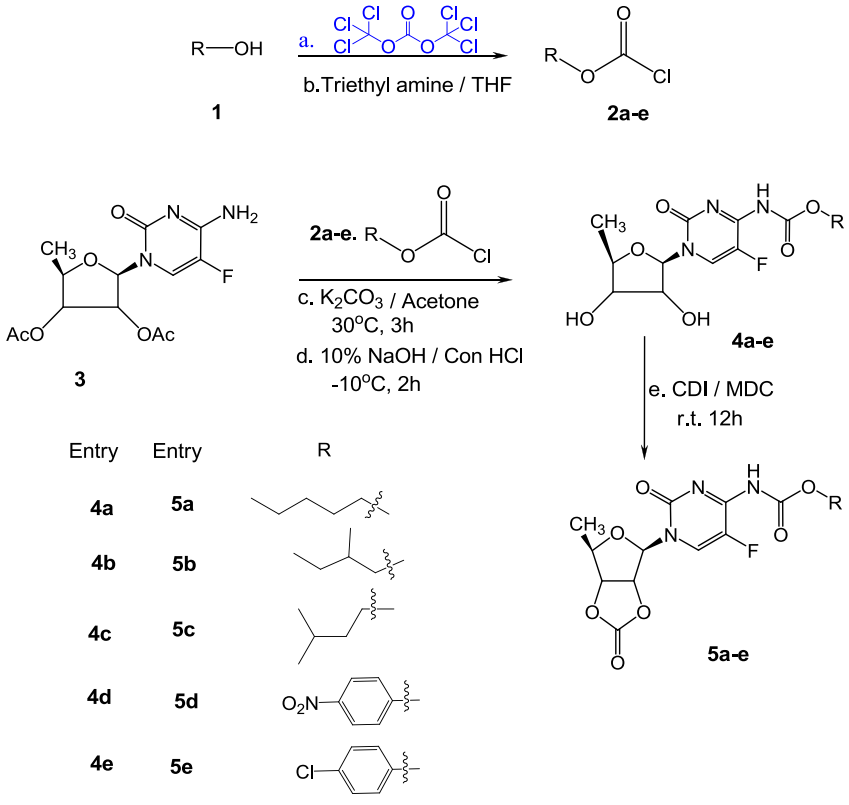
2. Chemistry

The synthesis of series of 5′-deoxy-5-fluorocytidine derivatives, cytidine-5′-deoxy-5-fluoro-N [(alkoxy/aryloxy)] carbonyl-cyclic 2′,3′-carbonates, was carried out by the following way. In the first step 2′,3′-di-*O*-acetyl-5′-deoxy-5-fluorocytidine was treated with various chloroformates in the presence of K_2CO_3 in acetone to obtain the intermediates **4a**–**e**. They were further reacted with carbonyl diimidazole (CDI) in methylene dichloride (CH_2Cl_2) to afford the title compounds **5a**–**e**.

The chemical structures of all the title compounds **4a**–**e** and **5a**–**e** were characterized by IR, ^1H, ^{13}C NMR and APCI-MS studies and their data are presented in the experimental section. Characteristic IR stretching absorptions were observed in the regions 3101–3235 cm^{-1} and 3402–3488 cm^{-1} for N–H [8], O–H [9], respectively. The ester carbonyl stretching frequency was observed in the range 1722–1746 cm^{-1}. In the ^1H NMR spectra of title compounds **4d**, **4e**, **5d** and **5e**, the chemical shifts of aromatic hydrogens of the phenyl ring appeared as doublets in the region δ 6.42–7.88 [9,10]. The N–H hydrogen resonated as a broad singlet in the region δ 10.65–11.25 [10]. The OH protons were observed as a singlet in the region 8.52–11.34 ppm ^{13}C NMR chemical shifts for title compounds were observed in their expected regions. The carbamide carbon (–CONH) gave signal at δ 153.6–155.6 [8] (Scheme 1).

3. Pharmacology

The bacterial reverse mutation assay was used to evaluate the mutagenic potential of 5′-deoxy-5-fluorocytidine derivatives, to induce gene mutations in the bacterial reverse mutation assay in comparison to solvent control according to the plate incorporation test (Trial I) and the pre-incubation test (Trial II) using histidine dependent auxotrophic mutants of *Salmonella typhimurium* (strains TA1535, TA1537, TA98, TA100 and TA102). Cytotoxicity of the 5′-deoxy-5-fluorocytidine derivatives **4b**–**e** and **5a**–**e** was examined by using cells of human lung (NCI-69), prostrate (PZ-HPV-7), breast (MCF-7), and cervical (HeLa) cancer cell lines and expressed in terms of inhibitory activity (IC_{50}, mg/mL) obtained by the MTT method. The results are summarized in Table 1. The *in vivo* antitumor activity of compounds **5d** and **5e** was investigated with murine leukemia L1210 and the results are listed in Fig. 1 [11]. The activity was evaluated in terms of mean survival time (MST). L1210 cell line for implantation was maintained at 37 °C under an atmosphere of 5% CO_2 in a 75 cm^2 culture flask and subcultured once or twice per week in RPMI 1640 medium containing 10% fetal



Scheme 1. Synthetic route.

Table 1
Cytotoxicities of new 5′-deoxy-5-fluorocytidine derivatives against tumor cells.

Compound	R	IC_{50} (mg/mL)[a]			
		NCI-69	PZ-HPV-7	MCF-7	HeLa
5-FU	–	0.28 ± 0.02	0.17 ± 0.018	0.69 ± 0.04	0.04 ± 0.002
4a	Pentyl	0.30 ± 0.015	0.16 ± 0.019	0.71 ± 0.053	0.05 ± 0.012
4b	2-Methyl-1-butyl	0.37 ± 0.01	0.25 ± 0.012	0.12 ± 0.01	0.09 ± 0.0023
4c	3-Methyl-1-butyl	0.37 ± 0.021	0.25 ± 0.037	0.12 ± 0.004	0.09 ± 0.0015
4d	Nitrophenyl	0.10 ± 0.005	0.09 ± 0.001	0.18 ± 0.02	0.01 ± 0.005
4e	Chlorophenyl	0.09 ± 0.01	0.07 ± 0.02	0.10 ± 0.02	0.03 ± 0.0011
5a	Pentyl	0.18 ± 0.002	0.14 ± 0.002	0.08 ± 0.0034	0.08 ± 0.0018
5b	2-Methyl-1-butyl	0.33 ± 0.01	0.20 ± 0.01	0.07 ± 0.0025	0.18 ± 0.007
5c	3-Methyl-1-butyl	0.31 ± 0.018	0.35 ± 0.012	0.10 ± 0.005	0.13 ± 0.0021
5d	Nitrophenyl	0.030 ± 0.008	0.006 ± 0.0012	0.006 ± 0.00051	0.03 ± 0.0019
5e	Chlorophenyl	0.003 ± 0.0012	0.003 ± 0.0015	0.030 ± 0.005	0.006 ± 0.0009

[a] Activities against tumor cells (NCI-69, human lung; PZ-HPV-7, human prostrate; MCF-7, human breast; HeLa, human cervical) were measured by MTT assay after 3 days of incubation.

bovine serum. The tumor cells were resuspended in PBS to a concentration of 1×10^7/mL. Similarly, L1210 cells (1×10^6/mL) were inoculated intraperitoneally (ip) into male BDF-1 mice on day 0. The test compounds **5d** and **5e** and the reference compound, capecitabine, were orally administered (po) 20 times for 4 weeks and the maximum doses were decided by considering acute toxicity.

4. Results and discussion

All the new 5′-deoxy-5-fluorocytidine derivatives **4** and **5** were assessed for its potential to induce gene mutations according to the plate incorporation test (experiment I) and the pre-incubation test (experiment II) using Ames tester strains TA1535, TA1537, TA98, TA100 and TA102 along with the different positive controls for different strains. The assay was performed with and without liver microsomal activation based on the results of the pre-experiment. Each concentration and the controls were tested in triplicate. The title compounds **4a**–**e** and **5a**–**e** were tested at the following concentrations 0.312, 0.625, 1.25, 2.5, 5 mg/plate, both in the presence of metabolic activation (+S9) and in the absence of metabolic activation (−S9). The results revealed that there was no positive mutagenic effect in strains TA1537, TA1535, TA98, TA100 and TA102 both in the presence (10% v/v S9 mix) and in the absence of metabolic activation at any of the tested dose level when compared with the concurrent vehicle control.

Trial II results have shown a negative effect in strain TA1537, TA1535, TA98, TA 100 and TA102 both in the presence (10% v/v S9 mix) and in the absence of metabolic activation at any of the tested dose level when compared with the concurrent vehicle control. From the results of this study, it is concluded that the new 5′-deoxy-5-fluorocytidine derivatives in all the concentrations in the presence and absence (10% v/v S9 mix) of metabolic activation, is non-mutagenic to *S. typhimurium* tester strains viz., TA1537, TA1535, TA98, TA100 and TA102 when tested under the specified conditions. The reference mutagens showed a distinct increase in induced revertant colonies in all the tester strains both in the presence as well as in the absence of metabolic activation, without showing cytotoxicity.

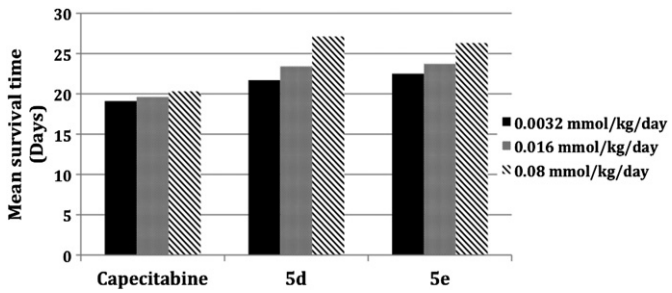


Fig. 1. MST time was measured after treating the L1210 cells implanted BDF-1 male mice with different concentrations of capecitabine, **5d** and **5e** compounds, 5 days a week for 3 weeks. **5d**, **5e** increases the mean survival time of mice compared with either with untreated control (18.2 days) or capecitabine treated group.

As shown in Table 1, all the new 5′-deoxy-5-fluorocytidine derivatives **4** and **5** were potent to inhibit the proliferation of NCI-69, PZ-HPV-7, MCF-7 and HeLa cells. Among all obtained compounds, **5d** and **5e** showed excellent inhibitory activity on the above cell lines, with IC_{50} values in the range of 0.003–0.030 mg/mL. It is evident that the *in vitro* potency of those bearing aromatic substituents (**5d**, R = *p*-nitrophenyl; **5e**, R = *p*-chlorophenyl) is better than that of those bearing alkyl and isoalkyl groups (**5b**, R = pentyl; **4b** and **5b**, R = 2-methyl-1-butyl, **4c** and **5c**, R = 3-methyl-1-butyl).

We initially evaluated that the *in vivo* activity of the new 5′-deoxy-5-fluorocytidine derivative **5d** and **5e** and the reference compounds capecitabine. The toxicity (LD_{50}) of **5d** and **5e** by oral administration was 2000 mg/kg for single dose and 200 mg/kg/day daily for 28 days in mice [13,14]. This value for the single dose is high compared to 115 mg/kg of 5-FU [15]. As shown in Fig. 1, the new 5′-deoxy-5-fluorocytidine derivatives **5d** and **5e** gave good antitumor activity over a broad dose range compared to capecitabine [16]. The compound **5d** and **5e** showed MST ranging from 23.4 to 23.7 at 0.16-mmol/kg/day to 27.1 and 26.3 days at 0.080 mmol/kg/day. Reference compound capecitabine show MST of 19.6 and 20.3 days at 0.16 and 0.08 mmol/kg/day dose, respectively. The above results clearly showed that compound **5d** increased the MST of the mice by 3.8 and 6 days at 0.16 and 0.08 mmol/kg/day dose, respectively, when compared with capecitabine treated mice. Evidently, the *in vivo* antitumor activity of **5d** and **5e** is better than that of capacitabine (Fig. 1). We also evaluated that the *in vivo* activity of the 5′-deoxy-5-fluorocytidine derivative **5a**, and the reference compounds capecitabine. Because of systemic toxicity we administered **5a** through po daily for two weeks, where as compounds **5d** and **5e** were administered 5 days a week for 3 weeks. As shown in Fig. 2, the antitumor activity of **5a** against L1210 leukemia in mice showed the best mean survival time value at high dose range, 17.3 days at 1.5 mmol/kg/day, after po administration compared to MST of capecitabine (12.8 days) [12].

Finally the correlation models generated among IC_{50} values and predicted log *P* values showed non linear graphs indicating that their correlation is not well significant. The values of *R* (coefficient of determination) and R2 (correlation coefficient) of the four correlation models are shown in Table 2 and the correlation models are shown in Fig. 3. The models are explaining that the two

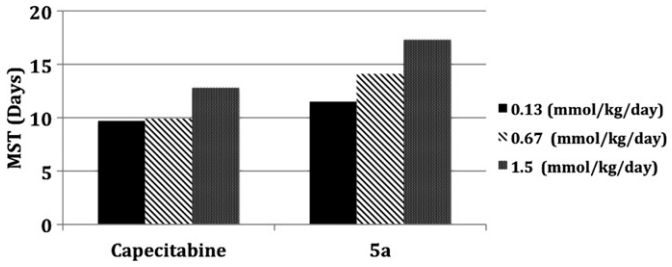


Fig. 2. MST time was measured after treating the L1210 cells implanted BDF-1 male mice with different concentrations of capecitabine, **5a** daily for two weeks. **5a** increases the mean survival time of mice compared with either with untreated control (8.9 days) or capecitabine treated group.

variables are not showing linear relationship and their cytotoxicity levels are independent among all the compounds.

5. Conclusion

In conclusion, we have synthesized the new 5′-deoxy-5-fluorocytidine derivatives **4** and **5** which show negative mutagenic activity in the bacterial reverse mutation test without inducing gene mutations by base pair changes or frame shifts in the genome of the strains used and show better antitumor activity than that of 5-FU or capecitabine. In particular, **5d** and **5e** show potent antitumor activities against L1210 leukemia cell line. The results provided a foundation for future design and development of antitumor compounds and also more potent drugs for cancer treatment.

6. Experimental protocols

6.1. Chemistry

Chemicals were purchased from Sigma–Aldrich, Merck and Lancaster, used as such without further purification. We procured the compound **3** (2′,3′-di-O-acetyl-5′-deoxy-5-fluorocytidine) from Hefei NodMed Pharmacy Co., Ltd. China. All solvents used for spectroscopy and other physical studies were reagent grade and were further purified by literature methods. Melting points (m.p.) were determined using a calibrated thermometer by Guna Digital Melting Point apparatus. Infrared spectra (IR) were recorded on a Nicolet 380 FT-IR spectrophotometer. Samples were recorded as potassium bromide (KBr) discs. ^{1}H and ^{13}C NMR spectra were recorded in DMSO-d_6 on a Bruker AMX 300 MHz spectrometer operating at 300 MHz for ^{1}H, 75 MHz for ^{13}C NMR and the chemical shifts values are expressed in parts per million (ppm) with reference to tetramethylsilane (TMS). LC mass spectra were recorded on a Jeol SX 102 DA/600 mass spectrometer.

6.1.1. Preparation of 2-methyl-1-butyl chloroformate

To the stirred solution of 2-methyl-1-butanol (1.474 g, 0.0167 mol) in dry tetrahydrofuran (20 mL) at nitrogen atmosphere, was added a solution of triphosgene (5 g, 0.0167 mol) at 0 °C in the presence of TEA (1.688 g, 0.0167 mol) over a period of 15 min. The reaction mixture was further stirred at room temperature 6 h, progress of the reaction was monitored by TLC (ethyl acetate: hexane 1:1). After completion of the reaction, it was filtered and washed with 2 volumes (10 mL) of THF to obtain the liquid compound 2-methyl-1-butyl chloroformate.

Table 2
Correlation analysis.

Tumor cell type[a]	R[b]	R2[c]
NCI-69	0.1166	0.0136
PZ-HPV-7	0.0774	0.0060
MCF-7	0.3029	0.0917
HeLa	0.3792	0.1438

[a] The type of tumor cells for which their respective IC_{50} values were taken for the construction of correlation models.
[b] Coefficient of determination.
[c] Correlation coefficients calculated from correlation analysis.

6.1.2. Preparation of 5′-deoxy-5-fluoro-N^4-(2-methyl-1-butyloxycarbonyl) cytidine [10]

To a solution of K_2CO_3 (0.626 g, 0.0453 mol) in acetone (80 mL) was added 2′,3′-di-O-acetyl-5′-deoxy-5-fluorocytidine (14 g, 0.0425 mol) in a 500 mL 4-necked RB flask fitted with magnetic bar, thermo pocket, guard tube and nitrogen inlet and stirred for 10–15 min 2-Methyl-1-butyl chloroformate first half (2.279 g, 0.0151 mol) was added through additional funnel over a period of 15–20 min at room temperature and continued to stirred for 3 h. After completion of 3 h, second half of 3-methyl-1-butyl chloroformate (1.129 g, 0.0075 mol) added at 25–30 °C, progress of the reaction was monitored by TLC (ethylacetate: hexane 1:1). After completion of the reaction, the mixture was filtered and washed with 2 volumes (50 mL) of acetone. To the acetone layer 4 volumes of 10% NaOH solution added at −10 to −15 °C over 2 h. After completion of the reaction indicated by TLC, the pH of the reaction mixture adjusted to 6.0 by using concentrated HCl. The acetone was distilled off completely by using rota evaporator. Thus obtained aqueous layer extracted with ethyl acetate 5 volumes (50 mL) and ethyl acetate layer reduced to 1 volume stirred for 30 min then 5 volumes of tertiary methyl butyl ether (TBME) (50 mL) was added, the obtained precipitate was stirred for 1 h at 20–25 °C and 2 h at 0–5 °C filtered off washed with 9:1 volumes of TBME and ethyl acetate and suck dried to obtained the crude product and it was further purified by column chromatography (1:1 hexane/EtOAc) to get the title compound.

6.1.3. Preparation of 5′-deoxy-5-fluoro-N^4-(2-methyl-1-butyloxycarbonyl) cytidine-2′,3′-carbonate

Methylene dichloride (120 mL) was taken in a 500 mL 4-necked RB flask fitted with magnetic bar, thermo pocket, guard tube and nitrogen inlet. 5′-Deoxy-5-fluoro-N^4-(2-methyl-1-butyloxycarbonyl)-cytidine (4 g, 0.0114 mol) was added in one lot, once the clear solution was formed, CDI (2.257 g, 0.01393 mol) was added in one lot under nitrogen atmosphere and the reaction mixture was stirred over 10–12 h. After the completion of the reaction (TLC), the reaction mixture was filtered and washed with 2 volumes (20 mL) of methylene dichloride. Thus obtained crude product was further purified by column chromatography (1:1 hexane/EtOAc) to get the title compound. The desired product was isolated and concentrated in rota evaporator. Finally it is dried under vacuum at 50 °C to get the pure product.

The same experimental procedure was adopted for the preparation of the remaining title compounds.

*6.1.3.1. Synthesis of 5′-deoxy-5-fluoro-N4-(n-pentyloxycarbonyl) cytidine (**4a**).* Yield: 90%; m.p. 119–121 °C; IR (KBr) 3488, 3210, 2975–2886, 1728, 1686 cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6) δ: 0.91–0.98 (m, 6H), 1.12–1.32 (m, 4H), 1.44–1.64 (m, 2H), 3.65 (q, J = 6.0 Hz, 1H), 3.89–4.05 (m, 4H), 5.06 (d, J = 6.0 Hz, 1H), 5.42 (s, 1H), 5.66 (d, J = 3.0 Hz, 1H), 8.06 (br s, 1H), 10.55 (br, s, 1H). ^{13}C NMR (75 MHz, DMSO-d_6) δ: 155.6, 129.8, 93.7, 81.0, 75.9, 71.8, 65.9, 38.5, 35.5, 26.9, 26.0, 22.9, 18.5, 16.6, 11.6. LCMS m/z (%) 360 $[M + H]^+$; Anal. Calcd. for $C_{15}H_{22}FN_3O_6$: C, 50.14; H, 6.17; N, 11.69%. Found: C, 50.08; H, 6.10; N, 11.63%.

*6.1.3.2. Synthesis of 5′-deoxy-5-fluoro-N^4-(2-methyl-1-butyloxycarbonyl) cytidine (**4b**).* Yield: 85%; m.p. 119–120 °C; IR (KBr) 3502, 3226,

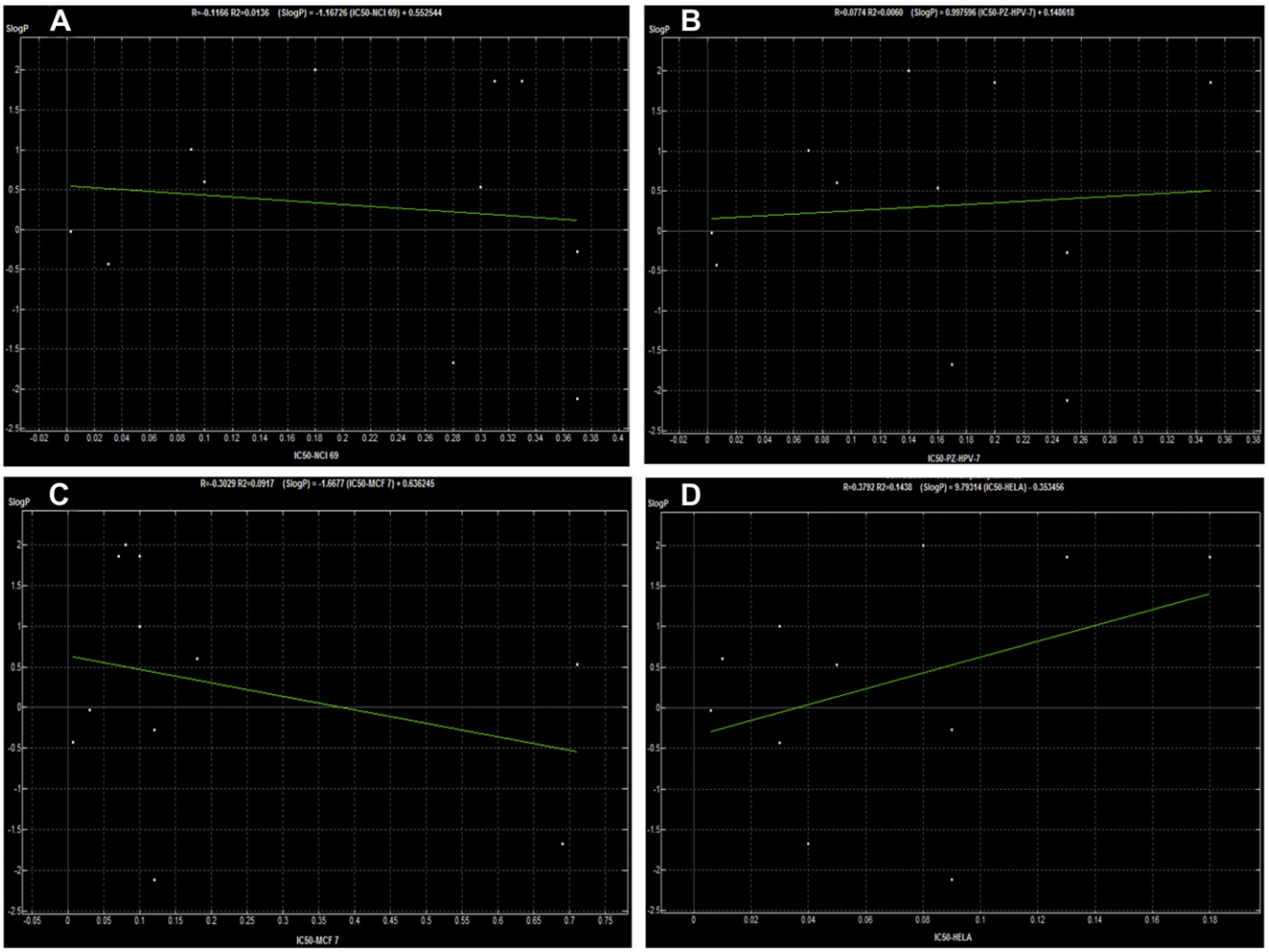


Fig. 3. Correlation plots generated for IC_{50} and log *P* values of the compounds. A. NCI-69, B. PZ-HPV-7, C. MCF-7 and D. HeLa cell types.

2966–2880, 1720, 1683 cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6) δ: 0.80–0.87 (m, 6H), 1.04–1.46 (m, 5H), 1.61–1.71 (m, 1H), 3.69 (q, J = 6.0 Hz, 1H), 3.84–4.16 (m, 4H), 5.21 (d, J = 3.6 Hz, 1H), 5.72 (d, J = 3.2 Hz, 1H), 5.80 (s, 1H), 7.85 (br, s, 1H), 10.21 (br, s, 1H). ^{13}C NMR (75 MHz, DMSO-d_6) δ: 155.4, 129.7, 93.7, 81.0, 75.9, 71.8, 50.0, 48.3, 35.5, 35.3, 26.9, 22.19, 19.6, 16.7, 11.6. LCMS m/z (%) 358 $[M-H]^-$; Anal. Calcd. for $C_{15}H_{22}FN_3O_6$: C, 50.14; H, 6.17; N, 11.69%. Found: C, 50.19; H, 6.13; N, 11.69%.

*6.1.3.3. Synthesis of 5′-deoxy-5-fluoro-N⁴-(3-methyl-1-butyloxycarbonyl) cytidine (**4c**).* Yield: 85%; m.p. 120–121 °C; IR (KBr) 3508, 3229–3121, 2965–2907, 1722, 1684 cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6) δ: 0.85–0.91 (m, 6H), 1.12–1.43 (m, 5H), 1.61–1.71 (m, 1H), 3.69 (q, J = 6.0 Hz, 1H), 3.66–3.99 (m, 4H), 5.25 (d, J = 3.1 Hz, 1H), 5.72 (d, J = 6.0 Hz, 1H), 5.80 (s, 1H), 7.84 (br, s, 1H), 9.32 (br, s, 1H). ^{13}C NMR (75 MHz, DMSO-d_6) δ: 155.6, 129.4, 93.9, 81.4, 75.2, 71.5, 51.5, 48.7, 35.8, 35.3, 26.5, 22.30, 19.8, 16.8, 11.9. LCMS m/z (%) 358 $[M-H]^-$; Anal. Calcd. for $C_{15}H_{22}FN_3O_6$: C, 50.14; H, 6.17; N, 11.69%. Found: C, 50.15; H, 6.14; N, 11.68%.

*6.1.3.4. Synthesis of 5′-deoxy-5-fluoro-N⁴-(4-nitro-phenyloxycarbonyl) cytidine (**4d**).* Yield: 87%; m.p. 147–148 °C; IR (KBr) 3458, 3235, 2986, 1728, 1684 cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6) δ: 1.32–1.46 (m, 3H), 3.70 (q, J = 6.2 Hz, 1H), 3.85–3.89 (m, 2H), 5.25 (d, J = 3.1 Hz, 1H), 5.68 (d, J = 3.0 Hz, 1H), 5.80 (s, 1H), 6.64 (d, 2H, J = 8.4 Hz), 6.67 (d, 2H, J = 8.0 Hz), 7.86 (br, s, 1H), 10.26 (br, s, 1H). ^{13}C NMR (75 MHz, DMSO-d_6) δ: 155.8, 155.3, 152.8, 152.0, 142.1, 140.3, 131.8, 128.1, 121.5, 125.3, 92.8, 83.2, 82.4, 76.5, 18.0. LCMS m/z (%) 411 $[M+H]^+$; Anal. Calcd. for $C_{16}H_{15}FN_4O_8$: C, 46.84; H, 3.68; N, 13.65%. Found: C, 46.86; H, 3.65; N, 13.62%.

*6.1.3.5. Synthesis of 5′-deoxy-5-fluoro-N⁴-(4-chloro-phenyloxycarbonyl) cytidine (**4e**).* Yield: 86%; m.p. 153–155 °C; IR (KBr) 3426, 3198, 2952, 1726, 1672 cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6) δ: 1.28–1.34 (m, 3H), 3.68 (q, J = 6.0 Hz, 1H), 3.85–3.91 (m, 2H), 5.26 (d, J = 3.0 Hz, 1H), 5.72 (d, 1H), 5.80 (s, 1H), 6.02 (d, 2H, J = 8.0 Hz), 6.42 (d, 2H, J = 8.0 Hz), 7.88 (br, s, 1H), 10.52 (br, s, 1H). ^{13}C NMR (75 MHz, DMSO-d_6) δ: 155.2, 154.3, 152.6, 152.1, 142.6, 140.4, 131.2, 126.8, 125.3, 121.4, 92.4, 83.4, 83.0, 75.2, 18.4. LCMS m/z (%) 400 $[M+H]^+$; Anal. Calcd. for $C_{16}H_{15}ClFN_3O_6$: C, 48.07; H, 3.78; N, 10.51%. Found: C, 48.01; H, 3.74; N, 10.47%.

*6.1.3.6. Synthesis of 5′-deoxy-5-fluoro-N⁴-(n-pentyloxycarbonyl)cytidine-2′,3′-carbonate (**5a**).* Yield: 88%; m.p. 142–143 °C; IR (KBr) 3103–3195, 2933–2958, 1803, 1749, 1630 cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6) δ: 1.12 (t, J = 6.0 Hz, 3H), 1.24–1.54 (m, 7H), 1.76 (t, J = 6.0 Hz, 2H), 4.25 (t, J = 6.0 Hz, 2H), 4.51–4.58 (m, 1H), 5.23 (m, 1H), 5.73 (m, 1H), 6.02 (d, 1H), 8.47 (d, J = 9.0 Hz, 1H), 11.12 (br, s, 1H). ^{13}C NMR (75 MHz, DMSO-d_6) δ: 153.6, 153.1, 152.6, 151.0, 138.6, 135.4, 130.9, 92.7, 83.2, 82.1, 65.4, 27.8, 27.3, 21.6, 18.8, 13.7. LCMS m/z (%) 384 $[M-H]^-$; Anal. Calcd. for $C_{16}H_{20}FN_3O_7$: C, 49.87; H, 5.23; N, 10.90%. Found: C, 49.86; H, 5.20; N, 10.92%.

*6.1.3.7. Synthesis of 5′-deoxy-5-fluoro-N⁴-(2-methyl-1-butyloxycarbonyl) cytidine-2′,3′-carbonate (**5b**).* Yield: 84%; m.p. 143–145 °C; IR (KBr) 3120, 2948, 1798, 1732, 1626 cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6) δ: 0.56–0.76 (t, J = 6.0 Hz, 3H), 1.00–1.23 (m, 7H), 1.34 (d, J = 6.0 Hz, 2H), 4.16 (t, J = 6.0 Hz, 2H), 4.83–4.87 (m, 1H), 5.36 (m, 1H), 5.64 (m, 1H), 6.87 (d, J = 6.2 Hz, 1H), 8.06 (d, J = 9.0 Hz, 1H), 10.65 (br, s, 1H). ^{13}C NMR (75 MHz, DMSO-d_6) δ: 154.1, 153.2, 152.8, 150.1, 138.8, 135.6, 130.7, 94.9, 83.7, 80.2, 66.0, 29.3, 27.6, 21.9, 17.4, 12.8. LCMS m/z (%) 384 $[M-H]^-$; Anal. Calcd. for $C_{16}H_{20}FN_3O_7$: C, 49.87; H, 5.23; N, 10.90%. Found: C, 49.88; H, 5.22; N, 10.89%.

*6.1.3.8. Synthesis of 5′-deoxy-5-fluoro-N⁴-(3-methyl-1-butyloxycarbonyl) cytidine-2′,3′-carbonate (**5c**).* Yield: 86%; m.p. 143–144 °C; IR (KBr) 3124–3195, 2928–2955, 1812, 1758, 1628 cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6) δ: 0.62–0.69 (m, 3H), 1.06–1.35 (m, 7H), 1.36–1.40 (d, 2H), 3.75–3.86 (t, J = 6.0 Hz, 2H), 4.10–4.16 (m, 1H), 4.81–4.85 (m, 1H), 5.33–5.42 (m, 1H), 5.68–5.82 (m, 1H), 8.07 (d, J = 6.0 Hz, 1H), 9.72 (br, s, 1H). ^{13}C NMR (75 MHz, DMSO-d_6) δ: 155.4, 152.9, 140.5, 137.2, 129.8, 93.7, 81.06, 75.9, 71.87, 65.9, 38.5, 26.9, 26.0, 23.7, 18.5, 11.6. LCMS m/z (%) 384 $[M-H]^-$; Anal. Calcd. for $C_{16}H_{20}FN_3O_7$: C, 49.87; H, 5.23; N, 10.90%. Found: C, 49.85; H, 5.21; N, 10.91%.

*6.1.3.9. Synthesis of 5′-deoxy-5-fluoro-N⁴-(4-nitro-phenyloxycarbonyl) cytidine-2′,3′-carbonate (**5d**).* Yield: 86%; m.p. 167–169 °C; IR (KBr) 3235, 2986, 1812, 1746, 1632 cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6) δ: 1.32 (d, J = 6.0 Hz, 3H), 4.09 (q, J = 6.0 Hz, 1H), 4.78 (m, J = 6.0 Hz, 1H), 5.31 (m, 1H), 5.74 (d, J = 7.2 Hz, 1H), 6.68 (d, 2H, J = 8.2 Hz, Ar-H), 6.96 (d, 2H, J = 8.2 Hz, Ar-H), 7.68 (br, s, 1H), 11.25 (s, 1H). ^{13}C NMR (75 MHz, DMSO-d_6) δ: 156.2, 153.6, 151.0, 138.6, 135.4, 131.6, 130.9, 128.6, 92.7, 83.2, 83.0, 82.2, 65.4, 31.2, 29.7, 28.9, 18.7. LCMS m/z (%) 435 $[M-H]^-$; Anal. Calcd. for $C_{17}H_{13}FN_4O_9$: C, 46.80; H, 3.00; N, 12.81%. Found: C, 46.78; H, 2.97; N, 12.79%.

*6.1.3.10. Synthesis of 5′-deoxy-5-fluoro-N⁴-(4-chloro-phenyloxycarbonyl) cytidine-2′,3′-carbonate (**5e**).* Yield: 86%; m.p. 157–159 °C; IR (KBr) 3152, 2958, 1810, 1752, 1630 cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6) δ: 1.35 (d, J = 6.0 Hz, 3H), 4.12 (q, J = 6.0 Hz, 1H), 4.81 (q, J = 6.0 Hz, 1H), 5.32 (d, J = 9.0 Hz, 1H), 5.68 (br, s, 1H), 6.02 (d, J = 8.4 Hz, 2H), 6.54 (d, J = 8.4 Hz, 2H), 8.11 (d, J = 9.0 Hz, 1H), 10.65 (s, 1H). ^{13}C NMR (75 MHz, DMSO-d_6) δ: 156.4, 153.6, 151.2, 138.6, 135.8, 131.1, 130.6, 128.6, 123.2, 94.1, 84.2, 83.2, 81.5, 65.4, 32.4, 29.4, 18.5. LCMS m/z (%) 426 $[M+H]^+$; Anal. Calcd. for $C_{17}H_{13}ClFN_3O_7$: C, 47.96; H, 3.08; N, 9.87%. Found: C, 47.94; H, 3.05; N, 9.85%.

6.2. Pharmacology

6.2.1. Bacterial reverse mutation assay

The assay was performed with and without liver microsomal activation. Each concentration, including the negative, vehicle and positive controls, were tested in triplicate. The test item was tested at the following concentrations: 0.312, 0.625, 1.25, 2.5 and 5 mg/plate, both in the presence of metabolic activation (+S9) and in the absence of metabolic activation (−S9) [14,17–19,20].

For each strain and dose level, including the controls three plates were used. The following materials were mixed in a test tube and poured onto the selective agar plates:

100 μL Test solution at each dose level, solvent, negative control or reference mutagen solution (positive control),
500 μL S9 mix (for test with metabolic activation) or S9 mix substitution buffer (for test without metabolic activation),
100 μL Bacterial suspension,
2000 μL Overlay agar.

In the pre-incubation assay 100 μL test solution, 500 μL S9 mix/S9 mix substitution buffer and 100 μL bacterial suspensions were mixed in a test tube and incubated at 37 ± 2 °C for 20 min. After pre-incubation 2.0 mL overlay agar was added to each tube. The mixture was poured on minimal agar plates.

After solidification the plates were incubated upside down for 48 h (approx) at 37 ± 2 °C in the dark.

6.2.2. MTT assay

The HeLa (cervical carcinoma), MCF-7 (breast carcinoma), NCI-69 (lung carcinoma), and PZ-HPV-7 (parostate carcinoma) cells (obtained from American Type Culture Collection ATCC, Rockville, MD, USA) were cultured as monolayers and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin and 100 mg/mL streptomycin in a humidified atmosphere with 5% CO_2 at 37 °C. The growth inhibition activity was assessed as described previously, according to the slightly modified procedure of the National Cancer Institute, Developmental Therapeutics Program [21]. The cells were inoculated onto standard 96-well micro titer plates on day 0. The cell concentrations were adjusted according to the cell population doubling time (PDT): 1×10^4/mL for HeLa, NCI-69 cell lines (PDT = 20–24 h), 2×10^4/mL for MCF-7 cell line (PDT = 33 h) and 3×10^4/mL for PZ-HPV-7 (PDT = 47 h). Test agents were then added in five dilutions (10^{-8}–5×10^{-6} mol/l) and incubated for a further 72 h. Working dilutions were freshly prepared on the day of testing. After 72 h of incubation, the cell growth rate was evaluated by performing the MTT assay, which detects succinate dehydrogenase activity in viable cells. Each test was performed in quadruplicate in three individual experiments. The results are expressed as IC_{50}, which is the concentration necessary for 50% of inhibition.

6.2.3. In vivo activities of new 5′-deoxy-5-fluorocytidine derivatives against murine leukemia L1210

L1210 cells were implanted intraperitoneally (ip) into BDF-1 male mice (6 weeks old) on day 0 and the mice were divided into several groups (8 mice per group) on day 1. The test compounds (capecitabine, **5d**, **5e**) were dissolved in saline and orally (po) administrated 5 days a week for 3 weeks. Because of systemic toxicity the test compound **5a** was dissolved in saline and administrated per os (po) daily for 2 weeks.

6.2.4. Construction of correlation models

Correlation models were constructed among IC_{50} values of the compounds and their respective predicted log P (partition coefficient) values. The three dimensional models were constructed for all the compounds (**5-FU** and **4a**–**5e**) in Molecular Operating Environment (MOE) working area [22]. The structures were energy minimized in MMFF94x force filed with implicit solvated environment at an RMS gradient of 0.05. The stabilized conformations were used to predict the log P values using the MOE descriptor calculator module. The resultant values were used to construct the correlation models taking IC_{50} values on X-axis and log P values on Y-axis.

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Glossary

5-FU: 5-Fluorouracil
5′-DFCR: 5′-deoxy-5-fluorocytidine
5′-DFUR: doxifluridine
APCI-MS: Atmospherics pressure chemical ionization-Mass spectrometry
CDI: Carbonyl diimidazole
CDM: Methylene dichloride
DPD: dihydropyrimidine dehydrogenase
FUPA: 5-fluoro-ureidopropionic acid
MST: Mean survival time
MTT: (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)
TBME: tertiary methyl butyl ether
UFT: Tegafur-Uracil