Novel Prodrugs of Tegafur that Display Improved Anticancer Activity and Antiangiogenic Properties

Dikla Engel,[†,‡] Abraham Nudelman,*[,†] Nataly Tarasenko,[‡] Inesa Levovich,[†,‡] Igor Makarovsky,[†] Segev Sochotnikov,[†] Igor Tarasenko,[‡] and Ada Rephaeli*[,‡]

Chemistry Department, Bar-Ilan University, Ramat Gan, 52900, Israel, and Felsenstein Medical Research Center, Sackler Faculty of Medicine, Tel Aviv University Beilinson Campus, Petach Tikva, 49100, Israel

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New and more potent prodrugs of the 5-fluorouracyl family derived by hydroxymethylation or acyloxymethylation of 5-fluoro-1-(tetrahydro-2-furanyl)-2,4(1*H*,3*H*)-pyrimidinedione (tegafur, **1**) are described. The anticancer activity of the butyroyloxymethyl-tegafur derivative **3** and not that of tegafur was attenuated by the antioxidant *N*-acetylcysteine, suggesting that the increased activity of the prodrug is in part mediated by an increase of reactive oxygen species. Compound **3** in an in vitro matrigel assay was found to be a more potent antiangiogenic agent than tegafur. In vivo **3** was significantly more potent than tegafur in inhibiting 4T1 breast carcinoma lung metastases and growth of HT-29 human colon carcinoma tumors in a mouse xenograft. In summary, the multifunctional prodrugs of tegafur display selectivity toward cancer cells, antiangiogenic activity, and anticancer activities in vitro and in vivo, superior to those of tegafur. 5-Fluoro-1-(tetrahydro-2-furanyl)-2,4(1*H*,3*H*)-pyrimidinedione (tegafur, **1**), the oral prodrug of 5-FU, has been widely used for treatment of gastrointestinal malignancies with modest efficacy. The aim of this study was to develop and characterize new and more potent prodrugs of the 5-FU family derived by hydroxymethylation or acyloxymethylation of tegafur. Comparison between the effect of tegafur and the new prodrugs on the viability of a variety of cancer cell lines showed that the IC_{50} and IC_{90} values of the novel prodrugs were 5–10-fold lower than those of tegafur. While significant differences between the IC_{50} values of tegafur were observed between the sensitive HT-29 and the resistant LS-1034 colon cancer cell lines, the prodrugs affected them to a similar degree, suggesting that they overcame drug resistance. The increased potency of the prodrugs could be attributed to the antiproliferative contribution imparted by formaldehyde and butyric acid, released upon metabolic degradation. The anticancer activity of the butyroyloxymethyl-tegafur derivative **3** and not that of tegafur was attenuated by the antioxidant *N*-acetylcysteine, suggesting that the increased activity of the prodrug is in part mediated by an increase of reactive oxygen species. Compound **3** in an in vitro matrigel assay was found to be a more potent antiangiogenic agent than tegafur. In vivo **3** was significantly more potent than tegafur in inhibiting 4T1 breast carcinoma lung metastases and growth of HT-29 human colon carcinoma tumors in a mouse xenograft. In summary, the multifunctional prodrugs of tegafur display selectivity toward cancer cells, antiangiogenic activity and anticancer activities in vitro and in vivo, superior to those of tegafur.

Introduction

The synthesis and biological activities of prodrugs of butyric acid having the general formula $Me(CH_2)_2COOCH(R)OR^1$, where R = H, Me, Pr, *tert*-Bu; R^1 = OC-alkyl, OC-Ar, and $P(O)(OEt)_2$, have been described.[1–3] These acyloxyalkyl prodrugs serve as molecular carriers for the efficient transport of butyric acid to the cells, leading to a significant increase in its potency.[4] The expected intracellular hydrolytic degradation products of the prodrugs are two carboxylic acids and an aldehyde (Scheme 1).

While studying these prodrugs, we discovered that the formaldehyde, released metabolically together with butyric acid, a histone deacetylase (HDAC[a]) inhibitor, plays a major and specific role in increasing the antineoplastic activity and is a critical antiproliferative factor in the induction of cancer cell differentiation and death.[1,3,5–7] These compounds specifically affect molecular events in the cancer cell, including changes in gene expression and induction of apoptosis, and thereby contribute significantly to the improvement of the anticancer activity of the parent drugs. While the accepted paradigm views formaldehyde as a highly toxic substance, in living cells it plays a vital role in fundamental biological pathways. Intracellular formaldehyde is formed by oxidative demethylation of *N*-, *O*-, and *S*-methylated compounds, xenobiotics, and by demethylation of DNA and histones. In the cells, formaldehyde is captured by 5′-methyl-tetrahydrofolate, which converts homocysteine to methionine and, by addition of ATP, it is converted into *S*-adenosyl-L-methionine (SAM), the universal methyl donor participating in synthesis of essential molecules in the cells.[8,9] Formation of adducts between DNA and anthracyclins, mediated by intracellular formaldehyde, has been extensively studied by others and us and has been shown to potentiate the anticancer

* To whom correspondence should be addressed. Phone: +972-3-531-8314(A.N.); +972-3-937-6126(A.R.). Fax: +972-3-531-8314 (A.N.); +972-3-922-8096 (A.R.). E-mail: nudelman@mail.biu.ac.il (A.N.); adarep@post.tau.ac.il (A.R.).

† Bar-Ilan University.

‡ Tel Aviv University Beilinson Campus.

[a]Abbreviations: HDAC, histone deacetylase; SAM, *S*-adenosyl-L-methionine; AN-1, butyroyloxymenthyl butyrate; AN-7, butyroyloxymethyl-diethyl phosphate; AN-9, pivaloyloxymethyl butyrate; Dox, doxorubicin; tegafur, 5-fluoro-1-(tetrahydro-2-furanyl)-2,4(1*H*,3*H*)-pyrimidinedione; 5-FU, 5-fluorouracil; SC, semicarbazide; SSAO, semicarbazide sensitive amine oxidase; HMTA, hexamethylenetetramine; NAC, *N*-acetylcysteine; ROS, reactive oxygen species; HUVEC, human umbilical vein endothelial cells; CEA, carcinoma embryonic antigen; $ES^{+/-}$, electron spray; CI^+, chemical ionization.

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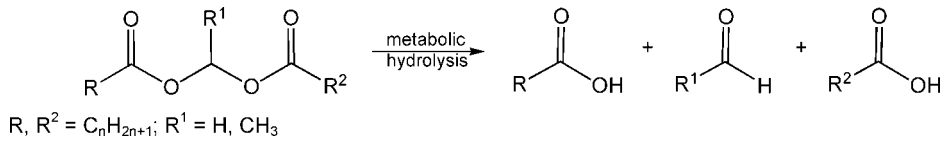
Scheme 1. Acyloxyalkyl Prodrugs and their Metabolites



activity of anthracyclines and to overcome drug resistance.[10–13] Synergy between formaldehyde releasing prodrugs and anthracyclines in killing of cancer cells has also been demonstrated.[12,13] In a recent study, we showed that the formaldehyde releasing prodrugs **AN-7** and butyroyloxymenthyl butyrate (**AN-1**), which synergize doxorubicin (Dox) anticancer activity, protect the heart against Dox toxicity.[14] The cellular formaldehyde released from the prodrugs was shown to be a dominant factor in the cardioprotective activity. The observations that the released formaldehyde exhibits selectively protect normal cardiomyocytes while it enhances killing of cancer cells suggests that it can add desirable properties to antineoplastic agents.

Although 5-fluorouracil (5-FU) is not orally bioavailable and cancer cells develop resistance to it, it has been one of the clinically used anticancer drugs for more than four decades.[15,16] 5-Fluoro-1-(tetrahydro-2-furanyl)-2,4(1*H*,3*H*)-pyrimidinedione (tegafur **1**), an orally active prodrug of 5-FU in mainly used for cancer treatment in combination with uracil (UFT) or leucovarin (S-1) in Japan. In a prospective study of 2934 node-negative breast cancer patients, tegafur plus uracil improved overall survival and it was suggested that UFT can be a useful and milder alternative to the cytotoxic adjuvant treatment for node-negative breast cancer or for elderly metastatic breast cancer patients.

To further improve the anticancer activity of tegafur, we synthesized 5-fluoro-1-(tetrahydrofuran-2-yl)-3-(hydroxymethyl)pyrimidine-2,4(1*H*,3*H*)-dione butyrate (**3**), 5-fluoro-1-(tetrahydrofuran-2-yl)-3-(hydroxymethyl)pyrimidine-2,4(1*H*,3*H*)-dione (**2**), and diethyl (5-fluoro-2,3-dihydro-3-(tertahydrofuran-2-yl)-2,6-dioxopyrimidin-1(6*H*)-yl)methyl phosphate (**5**) as novel multifunctional prodrugs of this antimetabolite (Table 1). Evaluation of these prodrugs on various types of sensitive and resistant cancer cells and their in vivo activity in a mouse syngeneic model are described.

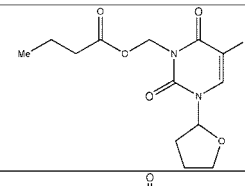
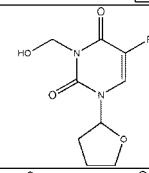
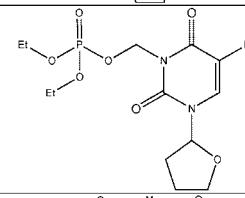
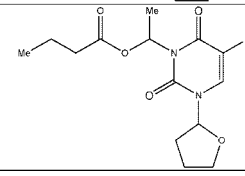
Results and Discussion

Effect of Tegafur (1) and its Derivatives on Viability of Cancer Cell Lines—Structure–Activity Relationships (SAR). Compounds that possess imido functional groups can react with formaldehyde to give stable *N*-hydroxymethyl derivatives and can also react with chloromethyl- or 1-chloroethyl butyrates to give the corresponding *N*-butyroyloxyalkyl derivatives. Thus, reaction of tegafur with formaldehyde, chloromethyl-, and 1-chloroethyl butyrates gave the corresponding prodrugs **2**, **3**, and **4** (Scheme 2).

The prodrugs we studied constitute a "drug combination treatment" because, upon metabolic degradation, they release 2–3 active anticancer molecules, for example, tegafur, formaldehyde, and butyric acid. A SAR study was conducted to assess the contribution of each metabolic component to the overall biological activity. For instance, to assess the importance of the metabolically released formaldehyde 5-fluoro-1-(tetrahydrofuran-2-yl)-3-(1′-hydroxyethyl)pyrimidine-2,4(1*H*,3*H*)-dione butyrate (**4**), where formaldehyde was replaced by acetaldehyde, was synthesized and evaluated (Table 1).

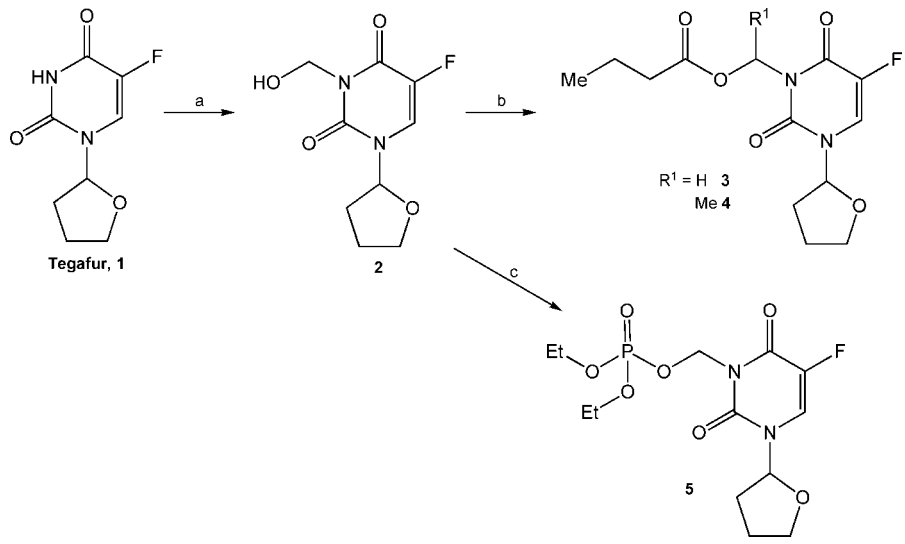
The effect of tegafur and its derivatives **3** and **2** that differ only in the butyric acid component (Table 1) on the viability

Table 1. Prodrugs and their Metabolites

Code	Structure	Metabolites
3		Tegafur, butyric acid, formaldehyde
2		Tegafur, formaldehyde
5		Tegafur, formaldehyde, diethyl phosphate
4		Tegafur, acetaldehyde butyric acid

of cancer cell lines of human (HT-29) and murine (CT-26) colon carcinoma, human and murine pancreatic carcinoma (BXPC-3 and panc02, respectively), and human Ewing sarcoma (SK-ES), is presented in Figure 1A,B. The viability was measured by the Hoechst assay and the average IC_{50} and IC_{90} values of at least three independent experiments were calculated from detailed titrations. The potency rank observed for the prodrugs was **3** > **2** >>> tegafur. For all the cell lines tested, the IC_{50} and IC_{90} values of tegafur were significantly (3–7-fold) higher than those of the derivatives. It should be noted that the IC_{90} values for tegafur were not reached experimentally, but were determined by extrapolation of the exponential titration curve, and the maximum cell killing was 40–80%, while the titration of the derivatives resulted in >90% cell killing. This difference is exemplified in the representative titration graphs of tegafur, **3**, and **2** in HT-29 cells (Figure 1C). In the majority of the tested cell lines (five out of nine), the IC_{50} values of **3** were significantly lower than those of **2** (Table 2). The IC_{50} and IC_{90} values of **3** were 35–50 and 75–150 μM, whereas those of **2** were 45–100 and 80–220 μM. Because the difference between **3** and **2** is the released butyric acid that possesses HDAC inhibitory activity, it is likely that the acid contributes to the improved anticancer activity of **3**. Although the mechanism by which the active metabolites of **3** act together is unknown, it was previously shown that butyroyloxymethyl-diethyl phosphate (**AN-7**) and pivaloyloxymethyl butyrate (**AN-9**) that concomitantly release butyric acid and formaldehyde potentiate the anticancer activity and act in synergy with chemotherapeutic anthracyclines.[17] Synergy between 5-FU and butyric acid in colon cancer has been reported in vitro and in vivo,[18–20]

Scheme 2. Synthesis of Prodrugs of Tegafur



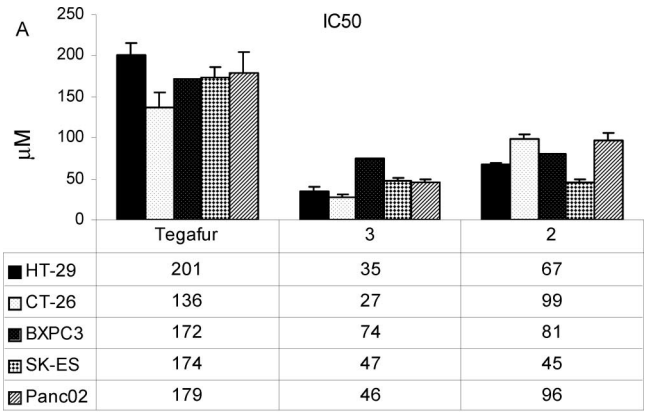
supporting the potentiation between tegafur and butyric acid observed in **3**.

To examine the contribution of formaldehyde in HT-29 human colon cancer cells, **3** that releases formaldehyde was compared to its analog **4** that releases acetaldehyde. The IC_{50} and IC_{90} values of **4** were 2–4.5 fold higher than those of **3**. Since these compounds differ only in the released aldehyde, it indicates a significant contribution by formaldehyde to the anticancer activity (Figure 2). These results support the notion that **3** is a multifunctional prodrug that undergoes metabolism in cells to release tegafur, formaldehyde, and butyric acid as active metabolites. Compound **2** was derivatized by addition of a diethyl phosphate functional group to give **5**, which showed a significantly improved activity over **2** and all other tested prodrugs. The IC_{50} and IC_{90} values of **5** were 19 and 80 μM, respectively. The diethylphosphate group, which improved aqueous solubility of the prodrug, could account in part for the increased potency. In previous studies, addition of this functional group has been found by us to increase anticancer potency by an as of yet unknown mechanism.[5]

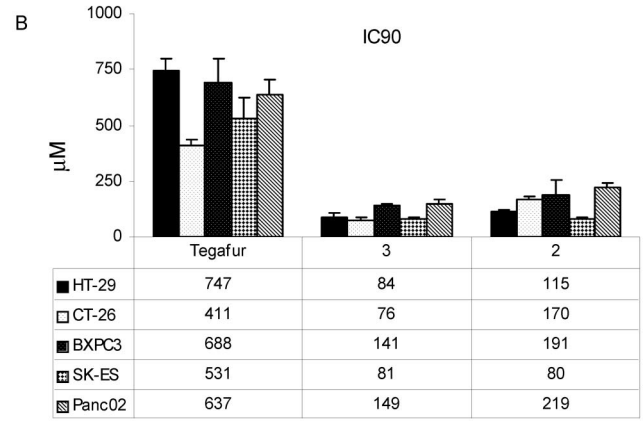
Comparison of the Effect of Tegafur and its Derivatives on Drug-Sensitive and -Resistant Cancer Cell Lines. The anticancer activities of tegafur,**3**, and **2** were compared in HT-29 and LS-1034, the sensitive and resistant human colon cancer, and in MES-SA and MES-DX, the sensitive and resistant human uterine sarcoma cell lines, respectively. LS-1034 cells displayed resistance to tegafur as evident by 4-fold greater IC_{50} and IC_{90} values compared to those of HT-29. The respective IC_{50} and IC_{90} values of tegafur in the sensitive HT-29 cells were 201 and 747 μM and in the resistant LS-1034 cells 519 and 1602 μM, displaying up to 4-fold greater values for the resistant line (Figure 3). In contrast, **3** and **2** showed similar effects on the viability of the resistant and the sensitive colon cells, implying that the tegafur-resistant cells were dramatically affected by the derivatives and these prodrugs overcame the mechanism of drug resistance. The uterine sarcoma MES-DX is a multidrug-resistant cell line established from MES-SA cells. MES-DX cells express high levels of mdr-1 mRNA and P-glycoprotein and exhibit marked cross resistance to a number of chemotherapeutic agents, such as vinblastine, vincristine, taxol, and additional cytotoxic drugs, but not to 5-FU. Consistent with these observations, the anticancer activity of tegafur toward MES-SA and MES-DX was similar.

Effect of Semicarbazide, a Sequestering Agent of Formaldehyde, on the Activity of 3 and 2. Semicarbazide, an antagonist of the enzyme semicarbazide-sensitive amine oxidase (SSAO), binds free cellular formaldehyde and blocks the production of formaldehyde from SSAO.[21] This copper-containing enzyme is found in abundance in blood plasma and vascular smooth muscle and its catalytic activity is elevated in the presence of some substrates, such as aminoacetone and methylamine. The enzyme is also involved in the production of formaldehyde in the cells and after deamination by SSAO methylglyoxal and formaldehyde are formed together with hydrogen peroxide as side products. In dose response studies, in the presence of 500 μM semicarbazide, the activities of **3** and **2** were attenuated in HT-29, LS-1034, CT-26, MES-SA, MES DX, and BXPC-3 cells. The calculated IC_{50} and IC_{90} values are shown in Figure 4. While semicarbazide at this concentration had no effect on cell viability, it inhibited the anticancer activity of the prodrugs, where the IC_{50} and IC_{90} values of **3** increased by 1.3–1.5-fold and those of **2** by about 2–2.5-fold. These results are indicative that formaldehyde released from **3** or from **2** plays an important role in the increased potency of the prodrugs. Furthermore, semicarbazide has a greater effect on **2**, which only releases formaldehyde, compared to its effect on **3**, which also releases butyric acid. The rationalization for the lesser impact of semicarbazide on **3** could be in part explained by the released butyric acid, unaffected by semicarbazide.

Effect of Formaldehyde Agonists on the Anticancer Activity of Tegafur. An additional approach to determine the contribution of the released formaldehyde to the anticancer activity is to pharmacologically increase its intracellular level and thereby emulate the activity of the prodrugs. To this end, the effect of tegafur in the presence or absence of the formaldehyde agonists, hexamethylenetetramine (HMTA) at a 250 μM concentration or succinic acid at a 2.5 μM concentration, was examined (Figure 5). HMTA hydrolyzes in cells to yield ammonia and formaldehyde.[22] Succinic acid inhibits mitochondrial aldehyde dehydrogenase and, as a result, formaldehyde accumulates in the cells.[23] Thus, these two agents enable us to study the effect of the released formaldehyde independently of the release of butyric acid or tegafur. HMTA and succinic acid at these concentrations did not affect the viability of tumor cell lines. In HT-29, BXPC-3, and CT-26



	Tegafur	3	2
HT-29	201	35	67
CT-26	136	27	99
BXPC3	172	74	81
SK-ES	174	47	45
Panc02	179	46	96



	Tegafur	3	2
HT-29	747	84	115
CT-26	411	76	170
BXPC3	688	141	191
SK-ES	531	81	80
Panc02	637	149	219

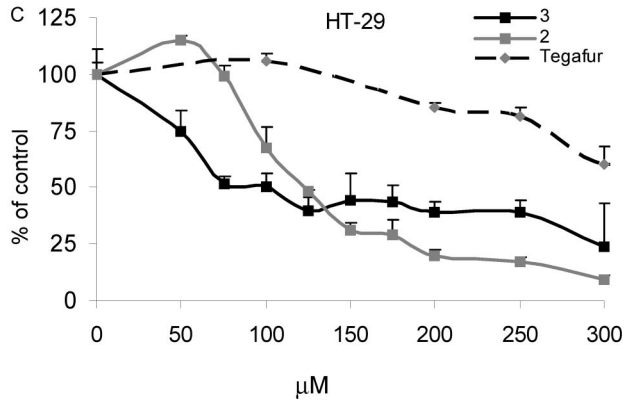


Figure 1. Effect of tegafur and its prodrugs on the viability of human cancer cells. Cells (5×10^3/well) were seeded in 96-well plates overnight and then treated with tegafur, **3**, and **2** for 72 h. Viability was determined by the Hoechst test and the IC_{50} (A) and IC_{90} (B) values were calculated. The experiments were performed in triplicates, where each IC_{50} value represents an average and standard error of three or more repeats. A representative titration of the drugs is shown in (C).

cells, these formaldehyde agonists did not affect the anticancer activity of tegafur, however, they augmented its activity in the resistant colon cancer cells LS-1034. The IC_{50} and IC_{90} values of tegafur in the presence of HMTA and succinic acid were 2–3-fold lower than those of tegafur as a single agent, suggesting that the increased intracellular formaldehyde level is responsible for the improved anticancer activity. In MES-DX, another cell line resistant to a variety of chemotherapeutic drugs but not to 5-FU, the anticancer activity of tegafur was unaffected by the addition of formaldehyde. The ability of formaldehyde to resensitize the cells to anthracyclines by increasing the amount of DNA-adducts was shown in other studies as a result of

Table 2. Comparative Activities of 3 and 2

cell line	3 IC_{50} (μM)	2 IC_{50} (μM)	*p*
CT-26	27 ± 4	99 ± 5	0.0002
Panc02	46 ± 3	96 ± 9	0.0002
LS-1034	42 ± 7	84 ± 8	0.015
U251	55 ± 10	103 ± 10	0.02
HT-29	35 ± 5	67 ± 2	0.021
MES-SA DXS	54 ± 5	63 ± 3	0.245
BXPC-3	81 ± 4	74 ± 9	0.5
SK-ES	47 ± 4	45 ± 4	0.7
MES-SA	71 ± 3	79 ± 5	0.77

synergy between **AN-9** or HMTA and doxorubicin.[22] The mechanism of the interaction between 5-FU and formaldehyde is unknown.

Activity of 3 but not that of Tegafur is Attenuated by the Antioxidant *N*-Acetylcysteine (NAC). We have shown that intracellularly released formaldehyde inhibits proliferation of cancer cells, induces cell death, and causes mitochondrial membrane collapse via a reactive oxygen species (ROS)-dependent mechanism (Levovich et al., submitted manuscript). Here we report that **3**, the formaldehyde releasing prodrug, and not tegafur inhibited proliferation of HT-29 and LS-1034 cells in a ROS-dependent manner. This conclusion is based on the observations that NAC reduced cytotoxicity and cell mortality and abolished the collapse of mitochondrial membrane potential caused by **3** (Figure 6A−C). In contrast, the activity of tegafur was not significantly altered by the addition of NAC. Cell death induced by 80 μM **3** and visualized using propidium iodide and Annexin V staining was abolished by NAC, while NAC had no significant effect on cell death induced by 400 μM tegafur

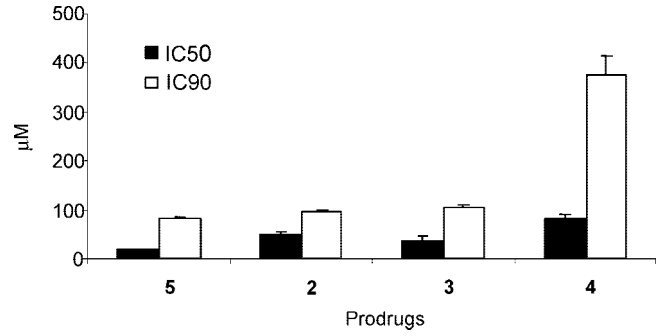
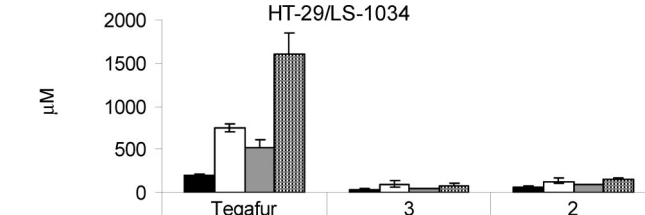
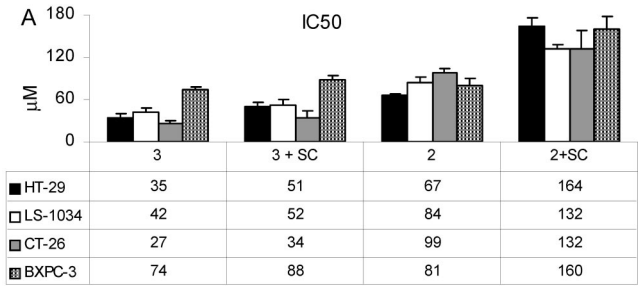


Figure 2. SAR study in human colon carcinoma cell line HT29. HT-29 cells (5×10^3 cells/well) in a 96-well plate were treated with **5**, **2**, **3**, and **4** for 72 h, viability was determined by the Hoechst assay, and IC_{50} and IC_{90} values were calculated.

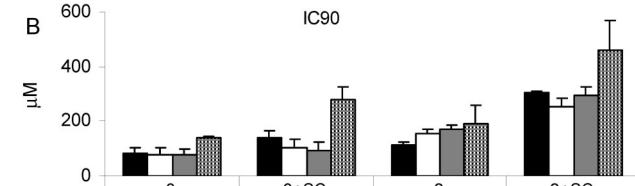


	Tegafur	3	2
IC50 HT-29	201	35	67
IC90 HT-29	747	84	115
IC50 LS-1034	519	42	84
IC90 LS-1034	1602	79	153

Figure 3. Effect of tegafur and its prodrugs on drug-sensitive and -resistant colon cancer cell lines. Comparison between the IC_{50} and IC_{90} values of sensitive HT-29 and resistant LS-1034 human colon carcinoma cells treated with tegafur, **3**, and **2**.

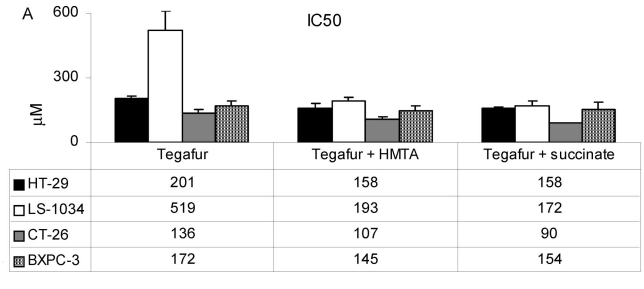


	3	3 + SC	2	2+SC
HT-29	35	51	67	164
LS-1034	42	52	84	132
CT-26	27	34	99	132
BXPC-3	74	88	81	160

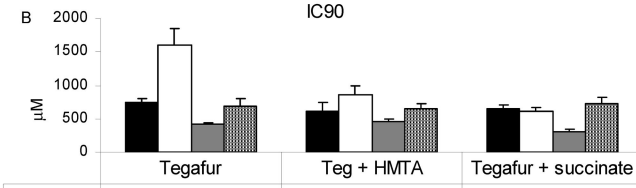


	3	3+SC	2	2+SC
HT-29	84	138	115	303
LS-1034	79	105	153	252
CT-26	76	94	170	296
BXPC-3	141	280	191	460

Figure 4. Effect of semicarbazide on the activity of **3** and **2**. The human sensitive HT-29, resistant LS-1034, BXPC-3, and murine CT-26 cell lines were treated with **3** and **2** with or without the addition of 500 μM semicarbazide. IC_{50} (A) and IC_{90} (B) values for viability by the Hoechst assay were determined.



	Tegafur	Tegafur + HMTA	Tegafur + succinate
HT-29	201	158	158
LS-1034	519	193	172
CT-26	136	107	90
BXPC-3	172	145	154



	Tegafur	Teg + HMTA	Tegafur + succinate
HT-29	747	612	644
LS-1034	1602	852	617
CT-26	411	464	311
BXPC-3	688	654	719

Figure 5. Effect of agonists of formaldehyde on the anticancer activity of tegafur. The human sensitive HT-29, resistant LS-1034, BXPC-3, and murine CT-26 cancer cell lines were treated with tegafur with or without the addition of succinic acid (2.5 mM) or HMTA (250 μM). IC_{50} (A) and IC_{90} (B) values for viability by the Hoechst assay were determined.

(Figure 6B). Loss of mitochondrial membrane potential ($\Delta\Psi_m$) is considered to be an early marker of apoptosis. The changes in $\Delta\Psi_m$ in HT-29 cells affected by **3** and tegafur were examined using the JC-1 dye, which exhibits a potential-dependent accumulation in mitochondria. Compound **3** (at 160 μM) caused significant $\Delta\Psi_m$ dissipation (Figure 6C) that was abolished by NAC. The $\Delta\Psi_m$ was unaffected by tegafur (up to 400 μM), NAC, or their combination. Taken together, these observations strongly suggest that **3**, which releases formaldehyde upon

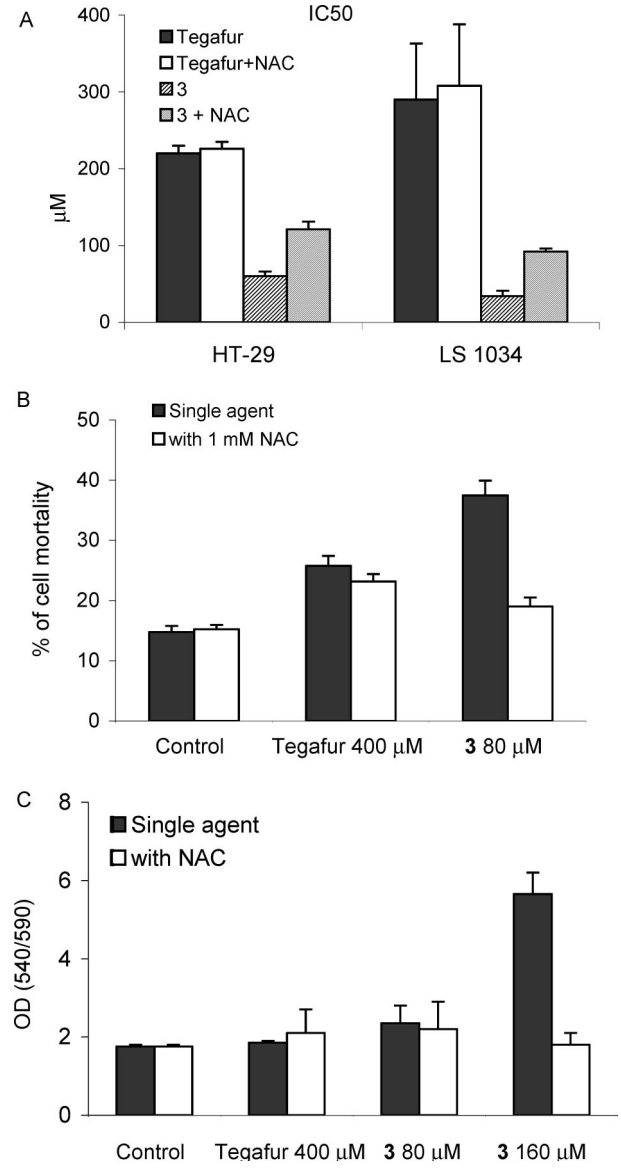
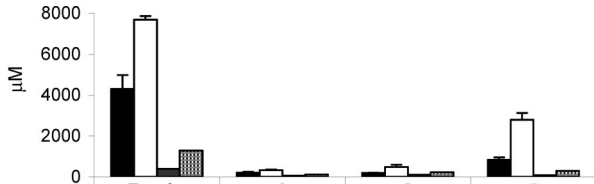


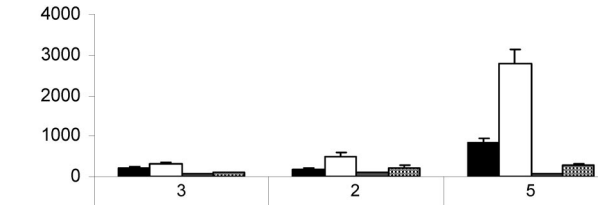
Figure 6. Activity of **3** is attenuated by NAC. (A) Cells (5×10^3) were seeded in 96-well plates and treated with the corresponding prodrugs with or without pretreatment with NAC. After a 72 h incubation, the cells were harvested and subjected to Hoechst assays. The data represent the mean values of more than three independent experiments. (B) HT-29 cells (2×10^5) were treated with tegafur or **3** for 24 h with or without pretreatment with 1 mM of the antioxidants NAC or glutathione (GSH) for 30 min, stained with propidium iodide and annexin V-FITC, and subjected to FACS analysis. (C) HT-29 cells (10^5/mL), seeded in a black 96-well plate, were treated with tegafur and **3** for 24 h with or without pretreatment with 1 mM NAC for 30 min. The mitochondrial membrane potential was determined by staining with the fluorescent probe JC-1.

metabolic degradation, induces cell death via a ROS-dependent mechanism involving mitochondrial loss of function.

Selectivity of the Prodrugs—Comparison of their Effect on the Viability of Glioblastoma and Normal Astrocyte Cells. To examine the selectivity of the prodrugs, the effect of tegafur and its derivatives on the viability of human normal astrocyte cells versus human glioblastoma cells, was compared (Figure 7). The IC_{50} and IC_{90} values of tegafur in the normal cells were 4.3 and 7.7 mM, while in glioblastoma cells they were 0.38 and 1.3 mM, respectively. Thus, the anticancer activity of tegafur was 6–11-fold higher against the glioblastoma cells. The anticancer activity in astrocyte cells compared to



	Tegafur	3	2	5
Ast IC50	4294	200	187	832
Ast IC90	7678	323	476	2786
U-251 IC50	384	55	103	73
U-251 IC90	1278	107	223	287



	3	2	5
Ast IC50	200	187	832
Ast IC90	323	476	2786
U-251 IC50	55	103	73
U-251 IC90	107	223	287

Figure 7. Comparison between the effect of tegafur and its prodrugs on the U251 glioblastoma cell line and normal astrocytes. The IC_{50} and IC_{90} values were determined by the Hoechst assay after 72 h of treatment.

glioblastoma cells, measured by IC_{50} and IC_{90} values were higher by 3–4-fold for **3**, 2-fold for **2**, and 10–11-fold for **5**. These results demonstrate the selectively of the prodrugs against cancer cells, where **5** exhibited the greatest selectivity. The selectivity of the prodrugs against cancer cells is an important factor that could be related to their low toxicity toward normal tissues.

Effect of Tegafur and its Prodrugs on Human Umbilical Vein Endothelial Cells (HUVEC) Tube Formation on Matrigel. The effect of tegafur and its prodrugs on the viability of HUVEC displayed IC_{50} and IC_{90} values for tegafur of 1.0 and 3.4 mM for **3** of 0.1 and 0.17 mM and for **2** of 0.07 and 0.12 mM, respectively (Figure 8). The effect of the prodrugs on the endothelial cells was similar to or lower than that displayed against cancer cells. The ability of tegafur and **3** to inhibit tube formation was examined on HUVEC (5×10^5 cells/well) seeded in 24-well plates and covered with a matrigel layer enriched with endothelial growth factors. After treatment with 80 μM tegafur or **3** for 3 and 8 h, tubes were visible only in the control and the tegafur treated cultures, whereas, in the **3**-treated ones, single cells with only a few small tubes were detected. After 24 h, only a few tubes remained in the tegafur-treated cultures and no tubes were visible in the **3**-treated cultures.

Compound 3 Possesses Histone Deacetylase Inhibitory Activity. Compound **3** that, upon metabolic hydrolysis releases the HDAC inhibitor butyric acid together with tegafur and formaldehyde, is expected to inhibit HDAC activity. To examine whether this inhibitory activity is effective in vivo, Balb/c mice bearing sc implanted 4T1 tumors were treated po tegafur or **3**. The level of histone acetylation in the tumors 4 h post-treatment was evaluated by Western blot analysis in tumor extracts of the sacrificed animals. An increase of over 2-fold in the acetylation level was observed (Figure 9A). These observations demonstrate that **3** reached the tumor site already after 4 h, and the released butyric acid inhibited the HDAC activity in the tumor, evidenced by the significant increase in histone H4 acetylation.

Effect of 3 in the Metastatic 4T1 Carcinoma Model. The in vivo anticancer activity of **3** was compared to that of tegafur in a syngeneic murine model of 4T1 breast carcinoma, considered to closely mimic stage IV of human breast cancer. The treatment group received po by gavage thrice a week saline or 50 mg/kg tegafur or an equimolar dose of **3** (75 mg/kg) for 3 weeks. The animals were then sacrificed and their lungs were examined for the appearance of metastatic lesions. The average number of metastases in the group treated with **3** was significantly lower than that of the tegafur-treated group and the latter was significantly lower than that of the control group (Figure 9B). The advantage of **3** in comparison to tegafur as an anticancer agent in vitro was also observed in vivo, showing that as an antimetastatic agent **3** is significantly better than that of tegafur.

Colon Carcinoma Xenograft Model. The effect of tegafur and **3** on tumor growth and survival was studied in the flank xenograft model of human colon carcinoma cells HT-29. In this accepted model for human colonic adenocarcinoma, 2 months old CD1 male nude mice were implanted sc with 2.5×10^6 HT-29 cells. When the tumors became palpable, the animals were treated po with 50 mg/kg tegafur and were compared to animals treated with an equimolar dose of **3**. Control mice were treated with vehicle only. Both drugs significantly inhibited tumor growth ($p < < 0.001$) but, at 50 mg/kg dose, **3** was significantly better than tegafur ($p < 0.05$; Figure 10A). In the HT-29 model, the serum level of the carcinoma embryonic antigen (CEA) is a reliable maker for advanced colon carcinoma and correlates with tumor burden. A comparison between the treated groups showed that both tegafur and **3** considerably decreased the CEA levels ($p < 0.05$), but the average CEA level of the **3** group was significantly lower than that of the tegafur group (Figure 10B).

Preliminary Toxicity. Dose escalation toxicity studies were performed with ICR mice. Doses of 100, 150, 200, 250, 500, and 750 mg/kg of tegafur and **3** were administered po to mice (11 or 12/group). One mouse in each group died after receiving 500 mg/kg (no gross pathological findings). Four days after receiving 750 mg/kg po, in the tegafur group, an additional animal died (total of 2). Two weeks later, two additional mice were dead in the tegafur group 4/11 (36%) and, in the **3** group, 3/12 (25%) animals died. Thus, the toxicity observed can be attributed to the tegafur and not to the released formaldehyde or butyric acid. Moreover, the therapeutic index of **3** allows a high safety margin.

It can be concluded that the advantage of the lead multifunctional prodrug **3** as an antineoplastic agent stems from its ability to target various functions in cancer cells, including ROS-sensitive activities affected by the formaldehyde, HDAC inhibitory functions affected by the butyric acid and cytotoxic activity elicited by the tegafur. An additional advantage of major consequence in cancer therapy is the ability of **3** to overcome multidrug resistance of tumor cells.

Experimental Section

Chemistry. Chemical shifts are expressed in ppm downfield from Me_4Si (TMS) used as internal standard. The values are given in δ scale. Multiplicities in the ^{13}C NMR spectra were determined by off-resonance decoupling. Mass spectra were obtained on a Q-TOF-micro from Micromass ($ES^{+/-}$, electron spray) and high-resolution mass spectra were obtained on a VG-Autospec-Fission from Micromass (CI^+, chemical ionization). Progress of the reactions monitored by TLC and flash chromatography were carried out on silica gel. All moisture-sensitive reactions were carried out in flame-

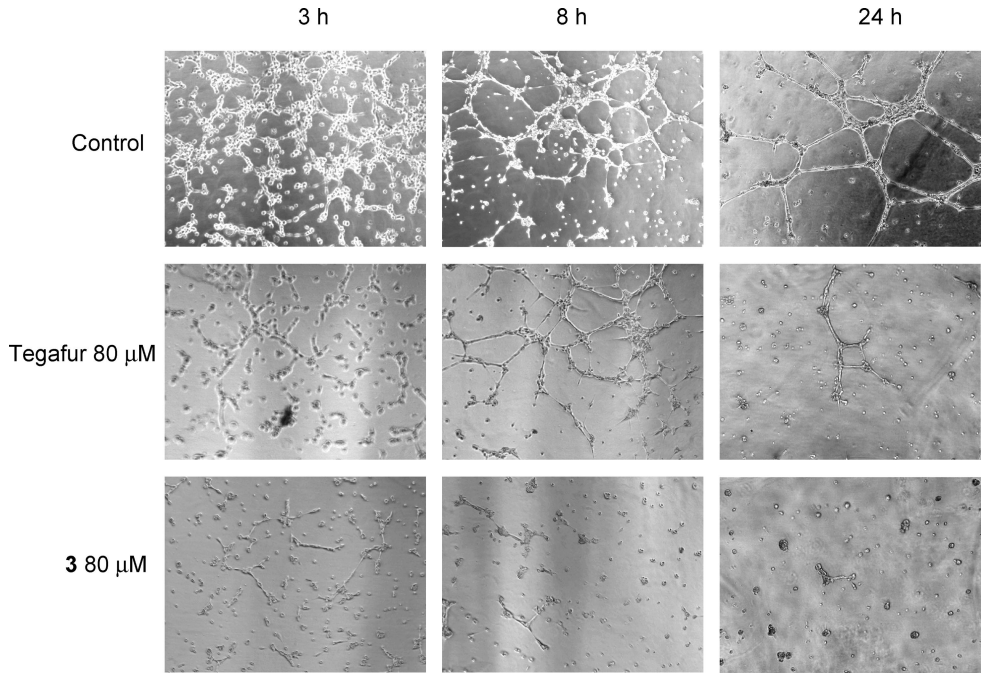


Figure 8. In vitro antiangiogenic effect of tegafur and **3** in HUVEC. Plates with 24 wells were precoated with 0.25 mL of matrigel per well. Each well was seeded with HUVEC (5×10^4) and incubated in supplemented EGM-2 medium containing 80 μM tegafur or 80 μM **3** for 3, 9, and 24 h each in triplicates. The cells were visualized using a light microscope and photomicrographs were taken at ×40 magnification. The picture presented is a representative of the triplicate treatment.

dried reactions vessels. Melting points were determined on a Fischer-Johns melting point apparatus.

(5-Fluoro-2,3-dihydro-3-((*R*)-tetrahydrofuran-2-yl)-2,6-dioxopyrimidin-1(6*H*)-yl)methyl butyrate, 3. To a solution of tegafur (0.26 g, 1.31 mmol) in DMF (2 mL) was added K_2CO_3 (0.18 g, 1.31 mmol), and the mixture was stirred for a few min followed by addition of chloromethyl butyrate (0.16 mL, 1.31 mmol, 1 equiv) in 1 mL of DMF over 15 min, and the mixture was stirred for 20 h at room temperature. Toluene (15 mL × 5) was added, and the organic layer was extracted with brine (15 mL × 5), dried over $MgSO_4$, and evaporated. The residual oil, dissolved in a minimal amount of EtOAc, was filtered through silica washed with EtOAc/hexane 3:1. The filtrate was evaporated and the residue was allowed to crystallize in the freezer to give off-white crystals of **3** (0.33 g, 83% yield), mp 63–64 °C. ^{1}H NMR (DMSO-d_6) δ 8.05 (d, J = 7.06 Hz, 1H, H-1), 5.95 (ddd, J = 6.3, 3.3, 1.5 Hz, 1H, H-2), 5.75 (s, 2H, H-6), 4.25 + 3.81 (dt, J = 8.4, 5.6 Hz, 1H + q, J = 8.4 Hz, 1H, 2H-5), 2.26 + 2.07 (m, 1H + m, 1H, 2H-4), 2.28 (t, J = 6.1 Hz, 2H, H-7), 1.95 (m, 2H, H-3), 1.51 (sext, J = 6.1 Hz, 2H, H-8), 0.85 (t, J = 6.1 Hz, 3H, H-9); ^{13}C NMR (DMSO-d_6) δ 171.8 (C-13), 156.0 (d, J = 26.4 Hz, C-11), 148.3 (C-12), 139.1 (d, J = 2.3 Hz, C-10), 125.0 (d, J = 33.2 Hz, C-1), 87.54 (C-2), 69.63 (C-6), 64.28 (C-5), 34.89 (C-7), 31.54 (C-3), 23.45 (C-4), 17.75 (C-8), 13.22 (C-9); MS (CI$^+$) m/z 301.124 ($C_{13}H_{18}N_2O_5F$, 100), 231.020 ([M − C_4H_7O]$^+$, 62); HRMS calcd for $C_{13}H_{18}N_2O_5F$ ([M + 1]$^+$, DCI/CH_4), 301.119975; found, 301.123656. Anal. Calcd for $C_{13}H_{17}N_2O_5F$ (301.11): C, 52.00; H, 5.71; N, 9.33. Found: C, 51.84; H, 5.72; N, 9.33.

5-Fluoro-1-((*R*)-tetrahydrofuran-2-yl)-3-(hydroxymethyl)pyrimidine-2,4(1*H*,3*H*)-dione, 2. A solution of tegafur (0.48 g, 2.4 mmol) and 35% aqueous formaldehyde solution (0.25 mL, 2.9 mmol, 1.2 equiv), was stirred for 50 min in a preheated oil bath at 50 °C. The mixture was then evaporated, and the colorless residual oil was dissolved in EtOAc and filtered through silica gel. The filtrate was evaporated almost to dryness and, upon addition of diethyl ether, the product was crystallized as white crystals (0.46 g, 84% yield), mp 168–170 °C. ^{1}H NMR (DMSO-d_6) δ (7.96 d, J = 6.6 Hz, 1H, H-1), 6.43 (t, J = 7.5 Hz, 1H, H-7), 5.96 (ddd, J = 6.3, 3.3, 1.5 Hz, 1H, H-2), 5.20 (d, J = 7.5 Hz, 2H H-6), 4.25 (dt, J = 7.8, 6.3 Hz, 1H, H-5), 3.82 (dt, J = 7.8, 7.2 Hz, 1H, H-5′), 2.26 (m, 1H, H-4), 2.03 (m, 1H, H-4′), 1.93 (m, 2H, H-3); ^{13}C NMR (DMSO-d_6) δ 156.2 (C-8), 148.7 (C-7), 139.4 (d, J = 299.1 Hz, C-9), 124.5 (C-1), 87.3 (C-2), 69.5 (C-6), 64.0 (C-5), 31.6 (C-3), 23.6 (C-4); ^{19}F-NMR (DMSO-d_6) δ −123.69 (ddt, J = 7, 1.6, 1 Hz). MS (CI$^+$) m/z 229.056 ($C_9H_{10}N_2O_4F$, 12), 202.0616 ([M − HCO]$^+$, 54.81), 131.016 ($C_4H_4N_2O_2F^+$, 28.94; C_4H_8O, 100). HRMS calcd for $C_9H_{10}N_2O_4F$ ([M − 1]$^+$, DCI/CH_4), 229.062460; found, 229.056193. Anal. Calcd for $C_9H_{10}N_2O_4F$ (230.19): C, 46.96; H, 4.82; N, 12.17. Found: C, 47.12; H, 4.95; N, 12.34.

Diethyl (5-Fluoro-2,3-dihydro-3-((*R*)-tetrahydrofuran-2-yl)-2,6-dioxopyrimidin-1(6*H*)-yl)methyl Phosphate, 5. To a solution of **2** (0.23 g, 0.98 mmol) in CH_2Cl_2 (5 mL), freshly distilled pyridine (0.6 mL, 4.9 mmol, 5 equiv), DMAP (0.18 g, 0.98 mmol, 1 equiv), and diethyl chlorophosphate (0.28 mL, 1.27 mmol, 1.3 equiv) were added. The mixture was stirred at room temperature until almost no starting material was spotted on TLC (EtOAc/hexane, 1:1, R_f = 0.1). The mixture was extracted with water and EtOAc and the organic phase was dried over $MgSO_4$ to yield after evaporation a yellowish oil that was chromatographed (EtOAc/hexane, 1:1). The first fraction contained tegafur, the second one contained **2**, and the third one contained **5** (0.35 g, 65% yield). ^{1}H NMR (DMSO-d_6) δ 8.04 (d, J = 6.6 Hz, 1H, H-1), 5.95 (ddd, J = 6.6, 3.6, 1.2 Hz, 1H, H-2), 5.65 + 5.62 (ddd, J = 9.0, 7.0, 0.5 Hz + ddd J = 9.0, 5.5, 0.5 Hz, AB system, 2H-6), 4.27 + 3.83 (dt, J = 7.8, 6.0 Hz, 1H, H-5 + q, J = 7.8 Hz, 1H, H-5), 4.04 (dq, J = 7.5, 1.75 Hz, 2H, H-7), 4.03 (dq, J = 7.5, 1.75 Hz, 2H, H-9), 2.27 + 2.04 (dtd, J = 13.2, 8.4, 6.6 Hz, 1H, H-4 + dddd, J = 13.2, 7.2, 6.3, 3.5 Hz, 1H, H-4), 1.93 (tt, J = 7.5, 5.5 Hz, 2H, H-3), 1.238 (td, J = 6.6, 0.6 Hz, 3H, H-8), 1.236 (td, 7.2, 0.6 Hz, 3H, H-10); ^{13}C NMR (DMSO-d_6) δ 155.8 (d, J = 27 Hz, C-11), 148.2 (C-12), 139.1 (d, J = 240 Hz, C-13), 125.1 (d, J = 30 Hz, C-1), 87.6 (C-2), 69.7 (C-5), 66.3 + 63.7 (dd, J = 3.0, 0.5 Hz + dd, J = 6.5, 1.6 Hz, C-7 + C-8), 64.8 (t, J = 3.0 Hz, C-6), 31.6 (C-3), 23.4 (C-4), 15.8 (d, J = 4.0 Hz, 2C-9); ^{31}P NMR (acetone-d_6) −2.86; MS (ES$^+$) m/z 389 ([M + Na]$^+$, 40.80), 367 (MH$^+$, 41.86), 297 ([MH − C_4H_7O]$^+$, 100); HRMS calcd for $C_{13}H_{21}FN_2O_7P$ (MH$^+$, DCI/CH_4), 367.1070; found, 367.1058.

1-(5-Fluoro-2,3-dihydro-3-(tetrahydrofuran-2-yl)-2,6-dioxopyrimidin-1(6*H*)-yl)ethyl Butyrate, 4. To a suspension of tegafur (0.32 g, 1.59 mmol) (3 mL) and K_2CO_3 (0.22 g, 1.59 mmol) in

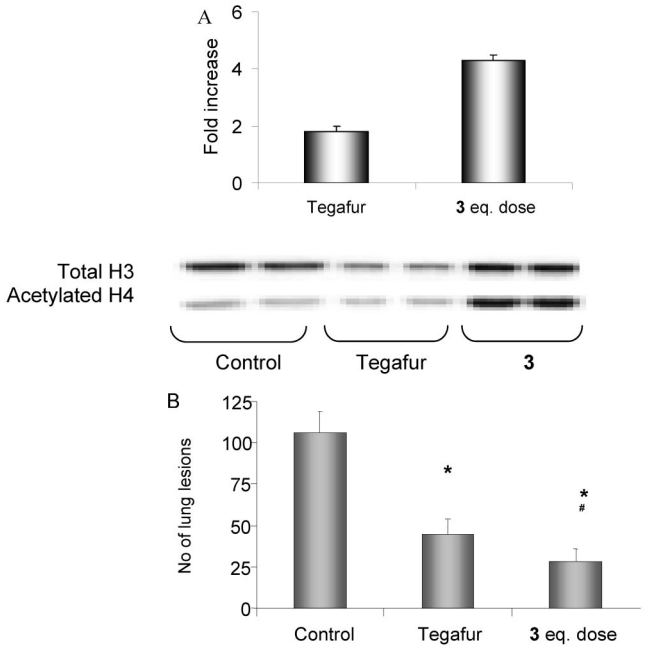


Figure 9. Inhibition by tegafur and **3** of (A) histone acetylation and (B) formation of metastatic lung lesions, in syngeneic murine models. (A) Balb/c mice were implanted sc with 5×10^4 murine 4T1 breast carcinoma cells. When the tumor reached a volume of 1.0 cm^3 the mice were treated po with saline, 100 mg/kg tegafur or **3**, and 4 h later, they were sacrificed. Histones were extracted from the tumor and 5 μg protein/well samples were run on 15% SDS gels and subjected to Western blot analysis with acetylated H4 antibody and with pan-H3 antibody as loading control. (B) Female Balb-c mice (10/group) were implanted sc with 4T1 breast carcinoma cells (5×10^4). When the tumor became palpable, tegafur (50 mg/kg) or equivalent doses of **3** were administered po thrice a week for three weeks. The animals were then sacrificed, their lungs were removed and stained with Bouin's solution, and the lung lesions were scored using a stereomicroscope. The asterisks indicate a significant difference between treated and untreated groups (*t*-test $p < < 0.05$) and # indicates a significant difference between **3** to tegafur ($p < 0.05$).

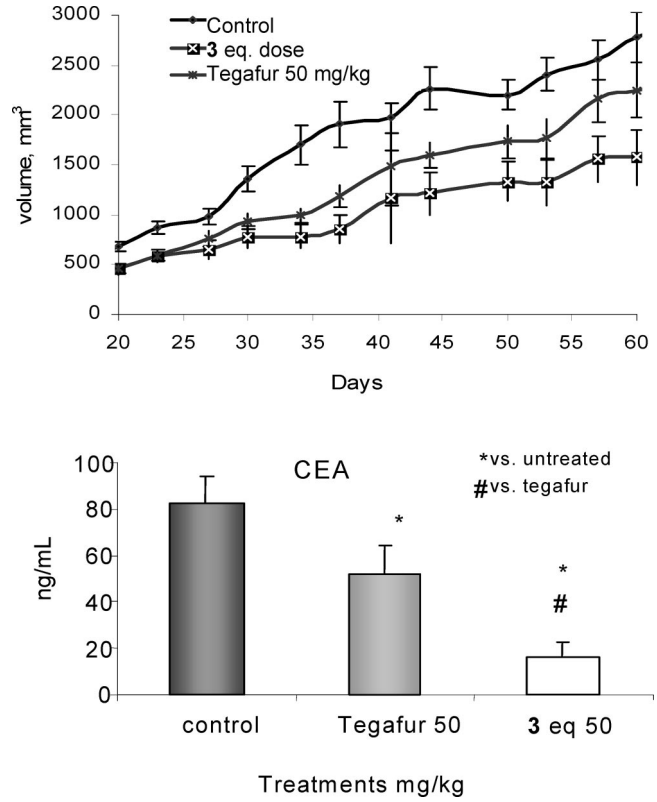


Figure 10. Compound **3** inhibits significantly better than tegafur the growth of sc implanted human colon carcinoma tumors. Two month old male, nude, CD1 mice were implanted sc with 2.5×10^6 HT-29 cells. When the tumors became palpable, the animals were divided, with 14 mice in the control and 12 animals in each of the treated groups.

DMF was added dropwise 1-chloroethyl butyrate (0.31 g, 1.98 mmol) over a period of 15 min. The milky mixture obtained was stirred at 80 °C overnight and monitored by TLC (EtOAc/hexane, 2:1) when no starting material could be detected. Toluene, water, and brine were added. The organic phase was extracted with brine (15 mL × 5), and the aqueous layer was extracted with toluene (15 mL × 5). The organic layers were combined, dried over $MgSO_4$, and evaporated. The residual oil was chromatographed (EtOAc/hexane, 1:1) to give after evaporation of the eluent **4** as a clear gel-like oil as a pair of two diastereomers in a ~1:1 ratio (0.1 g, 21% yield). The spectra were obtained for the diastereomeric mixture, which was not separated. ^{1}H NMR ($CDCl_3$) δ 7.92 + 7.92 (d, J = 6.6 Hz, 1H + d, J = 6.6 Hz, 1H, two sets of C*H*═CF), 7.07 (q, J = 6.0 Hz, 2H, two sets of NC*H*Me), 5.94–5.91 (m, 2H, two sets of N*H*O), 4.25 + 4.24 + 3.81 (dt, J = 8.1, 6.0 Hz, 2H, + dt, J = 8.1, 6.0 Hz, 2H, + q, J = 7.5 Hz, 2H, two sets of CH_2O), 2.65 (t, J = 7.2 Hz, 4H, two sets of CH_2—C═O), 2.28–2.18 + 2.07–1.96 (two m, 2H, two sets of CHCH_2), 1.95–1.87 (m, 4H, two sets of CH_2CH_2O), 1.69 (d, J = 7.2 Hz, 6H, two sets of *Me*CH), 1.51 (sext, J = 7.2 Hz, 4H, two sets of MeCH_2), 0.84 (t, J = 7.2 Hz, 6H, two sets of *Me*CH_2); ^{13}C NMR ($CDCl_3$) δ 171.6 (two sets of *C*O$_2$), 156.1 (d, J = 23.3 Hz, two sets of CF—*C*═O), 147.7 (two sets of N-*C*O), 139.0 (d, J = 22.8 Hz, two sets of *C*F), 124.0 + 124.1 (d, J = 38.5 Hz, two sets of ═*C*N), 87.3 + 87.2 (two sets of NCHO), 74.0 + 73.9 (two sets of NCHMe), 69.6 (two sets of *C*H$_2$O), 35.1 (two sets of *C*H$_2$C═O), 31.6 (two sets of *C*H$_2$CH—N), 23.5 (two sets of *C*H$_2$CH$_2$—CH—N), 17.7 (two sets of MeCH$_2$), 17.3 (two sets of *Me*CH), 13.2 (two sets of *Me*CH$_2$); MS (ES$^+$) *m/z* 353 ([M + K]$^+$, 1.77), 337 ([M + Na]$^+$, 100), 267 ([MH + Na − C_4H_7O]$^+$, 59.41).

Experimental Biology. Cell Cultures. Human colon cancer HT-29 (HTB-38) and LS-1034 (CRL-2158), uterine sarcoma MES-SA (CRL-1976) and MES-SA/Dx5 (CRL-1977), pancreatic carcinoma BXPC-3 (CRL-1687), Ewing sarcoma, SK-ES-1 (HTB-86), murine colon cancer CT-26 (CRL-2639), and breast cancer 4T1 (CRL-2539) cell lines were obtained from the American type Culture Collection (ATCC, Rockville, MD, U.S.A.). The human glioblastoma U251 cell line and astrocyte cells were obtained from Dr. Hass-Kogan (UCSF, U.S.A.). The cells were grown in RPMI-1640 medium supplemented with 10% heat inactivated fetal calf serum (FCS), 4.5 g/L glucose, penicillin (250 units/mL), streptomycin (125 mg/mL), 2 mM L-glutamine, and 10 mM HEPES at a pH 7.3. Human umbilical vein endothelial cells (HUVEC) and endothelial cell basal medium (EGM-2) were obtained from Clonetics (Rockland, ME, U.S.A.). The cells were grown in an incubator at 37 °C, 5% CO_2, and 98% humidity. Tissue culture materials were obtained from Biological Industries Beth-Haemek, Israel.

Treatment with the Prodrugs. For all the in vitro experiments, tegafur and its prodrugs (**3**, **2**, **5**, **4**) were solubilized in DMSO at a 0.5 M concentration, kept as aliquots of 100 mL at −70 °C, and diluted with medium before treatment so that the highest concentration of DMSO in the test medium was 0.1%. For the in vivo experiments, the drugs were solubilized in water before the treatment.

Viability Assay. Cell viability was measured using the Hoechst assay.[24] Cells at a density of 0.5–1 × 10^4 cells/well were seeded in tissue culture 96-well plates for 24 h, followed by exposure to different concentrations of the prodrugs (in triplicate) for 72 h, rinsed with PBS and fixed with 70% ethanol for 30 min. HUVEC cells were exposed to the drugs only for 48 h. The average IC_{50} values were determined by the best fitted curves (linear regression

or exponential function) of the cell survival percentage versus concentration of at least three independent experiments.

FACS Analysis. Cell survival and mortality were determined using a Mebcyto apoptosis kit (MBL-Japan). HT-29 cells were seeded in 60 mm^2 plates (2×10^5 cells), treated with the prodrugs for 24 h, washed with PBS, trypsinized and washed twice with ice-cold PBS. The cells were double-stained with annexin V-FITC and propidium iodide dissolved in the binding buffer according to the manufacturer's instructions and subjected to flow cytometry analysis (FACScalibur cytometer, Becton Dickinson).

Assessment of Mitochondrial Membrane Potential ($\Delta\Psi_m$). The fluorescent mitochondrial-specific cationic dye JC-1 was used to assess the status of $\Delta\Psi_m$ in cardiomyocytes after drug treatment. JC-1 underwent potential-dependent accumulation in the mitochondria. Mitochondria with a normal $\Delta\Psi_m$ concentrate the JC-1 dye in aggregated form, giving a red fluorescence. When the mitochondrial membrane is depolarized, the JC-1 formed monomers appearing as green fluorescence.[25] HT-29 cells (1×10^3) seeded on 96-well plates were treated for 24 h with the corresponding prodrugs. The cells were then washed with PBS and resuspended in 0.5 mL of 40 mM HEPES buffer, pH 7.4, supplemented with 0.65% NaCl and 4.5 g/L glucose (buffer A) at 37 °C containing 1 μg/mL JC-1. The cells were incubated for 15 min at 37 °C, washed, and resuspended in 200 mL of dye-free buffer A and added to a 96-well black plate and the fluorescence was measured immediately with a fluorescent plate reader using excitation/emission filters of 485/540 nm (green); 540/590 nm (red). The ratio of red/green fluorescence was calculated.

Evaluation of Tube Formation by Endothelial Cells. For this procedure fibrin gel in vitro angiogenesis assay kit (Chemicon, Germany) was used. The fibrinogen and thrombin solutions (supplied in the kit) were thawed and brought to 37 °C, 200 μL of the fibrinogen and 133 μL of the thrombin solutions were added to each well of a 24-well plate, and the plate was shaken and incubated for 15–60 min. HUVEC (5×10^4 cells) suspended in growth medium were seeded on the matrigel layer, and tegafur or **3** dissolved in medium were added for 3, 6, and 24 h. Cultures were visualized using a BX52 light microscope and images were taken with a digital camera.

Histone Acetylation In Vivo. For evaluation of in vivo histone acetylation, mice were treated po with vehicle or 100 mg/kg tegafur or **3** and sacrificed, and the tumors were removed and frozen immediately in liquid nitrogen and kept at −70 °C. The histones were purified and subjected to Western blot analysis, as described.[5] The fold increase of acetylated histones was determined by the ratio of band intensities of treated and untreated cells of mice, normalized to loading control (total histone H3). Rabbit antiacetylated histone H4, Lys-12 (Santa Cruz, U.S.A.), and pan (total) H3 (Cell Signaling, U.S.A.) were used for Western blot analysis. Horseradish peroxidase (HRP)-conjugated goat antirabbit IgG (Jackson ImmunoResearch Laboratories, U.S.A.) was the secondary antibody used.

4T1 Breast Carcinoma Metastatic Model. Balb-C mice were implanted sc with 5×10^4 4T1 murine breast carcinoma cells. When the tumor became palpable, the animals were divided into groups of 10 mice and treated po with 50 mg/kg tegafur thrice a week and with 75 mg/kg **3** (equimolar dose of 50 mg/kg of tegafur) for three weeks. After sacrificing the animals, their lungs and primary tumors were weighed, the lungs were fixed with Bouin's fixative solution, the metastases were counted, and the average number of metastases in each group and standard error were calculated.

Xenograft Flank Colon Carcinoma Model and Serum CEA. Male, 8−10 weeks old, nude CD1 mice (Harlan, Israel) were inoculated sc in the flanks with 5×10^6 HT-29 cells. Treatment commenced when the tumor was palpable (within 5–6 days). The mice were weighed and the tumors were measured with a caliper twice a week. Tumor volumes were calculated from measured lengths (L) and widths (W) using the $(L \times W^2)/2$ formula. At the end of the experiment, blood samples were drawn from the eyes of the mice (anesthetized slightly by inhalation of ether) or from the hearts of sacrificed animals. Blood was allowed to coagulate and centrifuged, and the collected serum was analyzed for CEA using an ELISA kit (MP Biomedical, Orangburg, NY).

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