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The cytostatic activity of NUC-3073, a phosphoramidate prodrug of 5-fluoro-2′-deoxyuridine, is independent of activation by thymidine kinase and insensitive to degradation by phosphorolytic enzymes

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ABSTRACT

A novel phosphoramidate nucleotide prodrug of the anticancer nucleoside analogue 5-fluoro-2′-deoxyuridine (5-FdUrd) was synthesized and evaluated for its cytostatic activity. Whereas 5-FdUrd substantially lost its cytostatic potential in thymidine kinase (TK)-deficient murine leukaemia L1210 and human lymphocyte CEM cell cultures, NUC-3073 markedly kept its antiproliferative activity in TK-deficient tumour cells, and thus is largely independent of intracellular TK activity to exert its cytostatic action. NUC-3073 was found to inhibit thymidylate synthase (TS) in the TK-deficient and wild-type cell lines at drug concentrations that correlated well with its cytostatic activity in these cells. NUC-3073 does not seem to be susceptible to inactivation by catabolic enzymes such as thymidine phosphorylase (TP) and uridine phosphorylase (UP). These findings are in line with our observations that 5-FdUrd, but not NUC-3073, substantially loses its cytostatic potential in the presence of TP-expressing mycoplasmas in the tumour cell cultures. Therefore, we propose NUC-3073 as a novel 5-FdUrd phosphoramidate prodrug that (i) may circumvent potential resistance mechanisms of tumour cells (e.g. decreased TK activity) and (ii) is not degraded by catabolic enzymes such as TP which is often upregulated in tumour cells or expressed in mycoplasma-infected tumour tissue.

1. Introduction

Current treatment of cancer using chemotherapeutics is largely based on the use of nucleoside analogues. These molecules are designed to mimic natural pyrimidine and purine nucleosides. After uptake by the cell, they are phosphorylated by cellular enzymes such as (deoxy)cytidine kinase (dCK), thymidine kinase (TK) and/or nucleo(s)(t)ide kinases. These antimetabolites can subsequently interfere with the *de novo* synthesis of DNA/RNA precursors to eventually inhibit DNA/RNA synthesis resulting in cytotoxic/static activity [1,2].

Fluoropyrimidine-based antimetabolites such as fluorouracil (5-FU), capecitabine and 5-fluoro-2′-deoxyuridine (5-FdUrd) are mainly used in the treatment of colon, breast and ovarian carcinoma [3–5]. Intracellularly, these drugs are metabolized to the monophosphate 5-FdUMP, which forms a stable inhibitory complex with thymidylate synthase (TS) and the reduced co-substrate 5,10-methylenetetrahydrofolate, thereby blocking binding of the normal substrate dUMP to the enzyme [6–8]. TS is the enzyme responsible for the conversion of dUMP to TMP and is therefore indispensable for cell proliferation, making it a crucial target for drug action. Among the fluoropyrimidines mentioned above, 5-FdUrd requires only one metabolic conversion, a phosphorylation catalysed by TK to generate 5-FdUMP [8]. This obligatory phosphorylation is often the rate-limiting step in the metabolism of many anti-cancer drugs (including 5-FdUrd), and is therefore still one of the limiting factors for the therapeutic use of nucleoside analogues. Hence, different strategies to improve the antitumour efficacy of nucleoside analogues are being investigated [2].

The charged nature of nucleoside monophosphates under physiological conditions results in poor, if any, penetration across the cell membrane [9]. They are also subject to extracellular dephosphorylation. Therefore, the direct administration of phosphorylated molecules to circumvent the first phosphorylation step has little therapeutic advantage. Hence, different strategies for bypassing the rate-limiting phosphorylation using various types of nucleoside 5′-monophosphate prodrugs for more efficient drug-delivery have been explored [10]. The administration of lipophilic phosphoramidate nucleotide prodrugs (ProTides) has proved successful for several molecules with anti-viral/cancer activity

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[11–13]. By masking the charges of the phosphate motif, good passive membrane diffusion of the prodrugs can be accomplished after which the prodrug is rapidly converted intracellularly into the nucleoside monophosphate by enzymatic cleavage [9].

Mycoplasmas are the smallest known self-replicating organisms and are characterized by the lack of a cell wall and a strongly reduced genome (600–1200 kb). Many of these bacteria have a parasitic lifestyle and reside in the human body causing asymptomatic infections [14]. It was shown that these prokaryotes tend to preferentially colonize tumour tissue: Huang et al. reported that 40–56% of human gastric, colon, oesophageal, lung and breast cancers are infected with mycoplasmas compared to 21–30% in non-tumourigenic tissue [15]. Pehlivan et al. found 22% of small cell lung cancer tissue samples and >80% kidney tissue samples of patients suffering renal cell carcinoma to be infected with mycoplasmas compared to 5% and 14%, respectively, in control tissue samples [16,17]. Chan et al. reported a 59% mycoplasma infection rate in ovarian cancer tissues [18] and other studies also reported a high infection rate in gastric [19,20] and cervical condyloma tissues [21]. Due to their reduced set of genes, mycoplasmas lack the pathway for *de novo* pyrimidine and purine synthesis and therefore express a wide array of salvage nucleo(s)(t)ide-metabolizing enzymes, such as thymidine phosphorylase (TP), deoxycytidine deaminase, etc. [22–25]. Already in 1985 it was observed that mycoplasma-encoded enzymes (e.g. TP), present in contaminated cell cultures, lead to decreased dTTP incorporation in lymphocytes [26]. Recently, it has been demonstrated that these enzymes, in particular the mycoplasma-encoded TP, can also interfere with the cytostatic activity of several chemotherapeutics, including 5-trifluorothymidine, *in vitro* [27–29]. Therefore we hypothesized that the elimination of mycoplasmas by antibiotics, suppression of mycoplasma-encoded enzymes in human tumour tissue or the development of mycoplasma-insensitive nucleoside analogue prodrugs may optimize treatment of cancer patients using purine and pyrimidine antimetabolites [29]. In the absence of such approaches, cancer patients may receive sub-optimal chemotherapeutic treatment.

This study was aimed at the development and assessment of TK-independent phosphoramidate prodrugs of 5-FdUrd that would also be insensitive to the TP-dependent inactivation of its free nucleoside analogue. From a wide variety of newly synthesized phosphoramidate prodrugs of 5-FdUrd, NUC-3073 (Fig. 1) was chosen for further in depth studies. This molecule contains a naphthyl and benzylalaninyl group to mask the charged 5′-phosphate on 5-FdUMP.

2. Materials and methods

2.1. Compound synthesis

The compound NUC-3073 (Fig. 1) (**1**) has been synthesized using phosphorochloridate chemistry as previously reported by

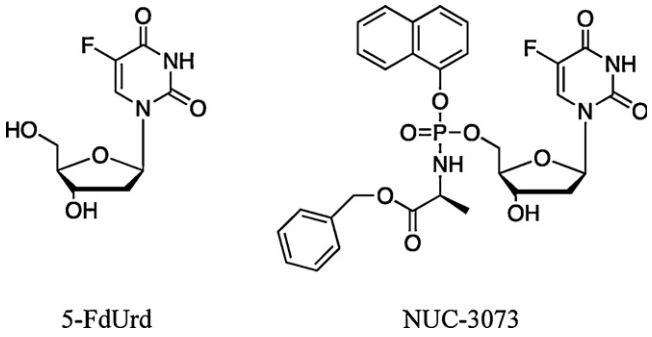
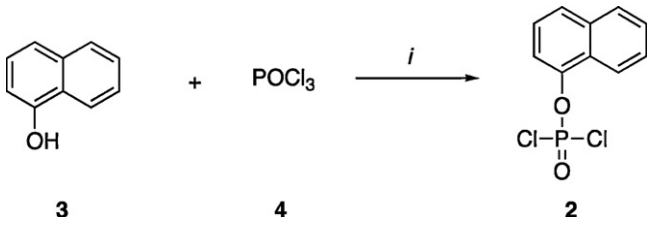


Fig. 1. Structural formula of 5-FdUrd and its phosphoramidate prodrug NUC-3073.



3 **4** **2**

Scheme 1. *Reagents and conditions*: (i) dry Et_2O, dry Et_3N, −78 °C, 30 min then R.T., 3 h.

McGuigan et al. [30–32]. Arylphosphorodichlorophosphate (**2**) has been prepared by coupling commercially available 1-naphthol (Sigma–Aldrich, Dorset, UK) (**3**) with phosphorus oxychloride (Sigma–Aldrich) (**4**) in the presence of Et_3N (Sigma–Aldrich) (Scheme 1) and this was allowed to react with L-alanine benzyl ester tosylate (NovaBiochem (now Merck Chemicals Ltd, Darmstadt, Germany)) (**5**) in the presence of Et_3N to generate the phosphorochloridate derivative (**6**) (Scheme 2).

The nucleoside 5-FdUrd (Fig. 1) (Carbosynth Ltd, Berkshire, UK) (**7**) was converted to the 5′-ProTide derivative by coupling with the phosphorochloridate derivative (**6**) in THF (Sigma–Aldrich) in the presence of *N*-methyl imidazole (NMI) (Sigma–Aldrich) to give the target compound NUC-3073 (**1**) (Scheme 3). The product was obtained as a mixture of two diastereoisomers as confirmed by the presence of two peaks in the ^{31}P and ^{19}F NMR spectra, and two closely spaced peaks detectable by HPLC.

Alternatively, the compound **1** was prepared using *t*BuMgCl (Sigma–Aldrich) (1 M solution in THF). Due to the lack of selectivity towards the primary hydroxyl group, formation of 3′-*O*-phosphorylated derivative **8** was also observed (Scheme 4).

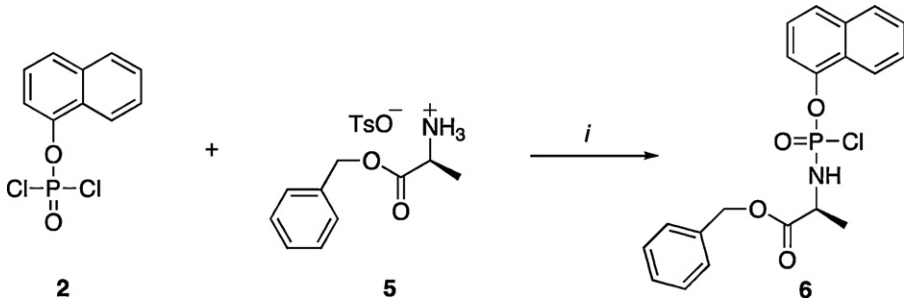
2.1.1. General

Anhydrous solvents were obtained from Sigma–Aldrich and used without further purification. All reactions were carried out under an argon atmosphere. Reactions were monitored with analytical TLC on Silica Gel 60-F254 precoated aluminium plates (Merck Chemicals Ltd) and visualized under UV (254 nm) and/or with ^{31}P NMR spectra. Column chromatography was performed on silica gel (35–70 μM). Proton (^1H), carbon (^{13}C), phosphorus (^{31}P) and fluorine (^{19}F) NMR spectra were recorded on a Bruker Avance 500 spectrometer (Bruker UK, Coventry, UK) at 25 °C. Spectra were auto-calibrated to the deuterated solvent peak and all ^{13}C NMR and ^{31}P NMR were proton-decoupled. Analytical HPLC was conducted by a Varian Prostar (LC Workstation-Varian prostar 335 LC detector) using Varian Polaris C18-A (Agilent Technologies, Edinburgh, UK) (10 μM) as an analytic column.

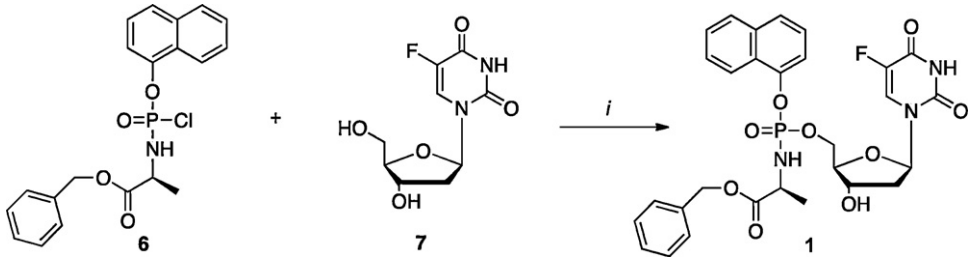
Low and high resolution mass spectra were performed as a service by Birmingham University, using electrospray (ES). CHN microanalysis was performed as a service by MEDAC Ltd, Surrey.

2.1.2. Synthesis of 1-naphthyl dichlorophosphate (2)

Phosphorus oxychloride (1.94 mL, 20.81 mmol) was added to a solution of 1-naphthol (3.00 g, 20.81 mmol) in diethyl ether (Sigma–Aldrich) (70 mL) under argon atmosphere, then anhydrous triethylamine (2.9 mL, 20.81 mmol) was added dropwise at −78 °C and the resulting reaction mixture was stirred for 30 min. Subsequently the reaction mixture was allowed to slowly warm up to room temperature for 3 h. A formation of the desired compound (**2**) was monitored by ^{31}P NMR. After the reaction was completed, the resulting mixture was filtered and then evaporated *in vacuo* under nitrogen to afford the crude colorless oil as product, which was used without further purification in the next step (4.59 g, 84%) [R_f = 0.93 (hexane–EtOAc (Fisher Scientific, Leicestershire, UK), 1:1)], ^{31}P NMR (202 MHz, $CDCl_3$): δ_P 5.07; ^1H NMR (500 MHz, $CDCl_3$): δ_H 7.52–7.71 (m, 4H, Ar*H*), 7.86–7.89 (m, 1H, Ar*H*), 7.95–7.98 (m, 1H, Ar*H*), 8.16–8.19 (m, 1H, Ar*H*).



Scheme 2. *Reagents and conditions*: (i) dry Et_3N, dry CH_2Cl_2, −78 °C, 30 min then R.T., 1 h.



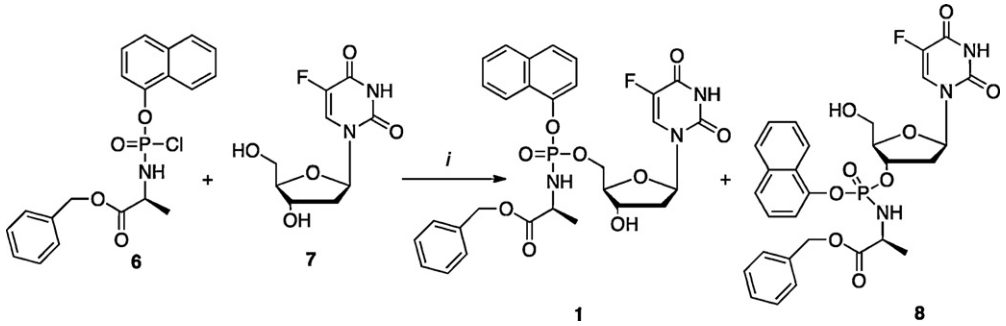
Scheme 3. *Reagents and conditions*: (i) NMI, dry THF, then phosphorochloridate (**6**), R.T., 16 h.

*2.1.3. Synthesis of 1-naphthyl-(benzyl-L-alaninyl) phosphorochloridate (**6**)*

Anhydrous triethylamine (2.66 mL, 19.14 mmol) was added dropwise under argon atmosphere at −78 °C to a stirred solution of 1-naphthyl dichlorophosphate (**2**) (2.50 g, 9.57 mmol) and L-alanine benzyl ester p-tosylate salt (NovaBiochem) (3.36 g, 9.57 mmol), in anhydrous DCM (Sigma–Aldrich) (50 mL). The reaction mixture was stirred at −78 °C for 30 min and then allowed to slowly warm to room temperature and stirred for 1 h. A formation of the desired compound was monitored by ^{31}P NMR. After the reaction was completed, the solvent was removed under reduced pressure and the crude residue was purified by flash chromatography (hexane/EtOAc 7/3) to give the product (**6**) as an oil (1.82 g, 47%) [R_f = 0.90 (hexane–EtOAc, 7:3)], ^{31}P NMR (202 MHz, $CDCl_3$, mixture of diastereoisomers): δ_P 7.92, 8.14 (Int.: 1.00:1.00); ^1H NMR (500 MHz, $CDCl_3$, mixture of diastereoisomers with a ratio of 1:1): δ_H 1.42–1.45 (m, 3H, $CHCH_3$), 4.20–4.23 (m, 1H, $CHCH_3$), 4.78–4.81 (m, 1H, NH), 5.09 (s, 2H, OCH_2Ph), 7.09–7.73 (m, 11H, ArH), 7.97–8.12 (m, 1H, ArH).

*2.1.4. Synthesis of 5-fluoro-2′-deoxyuridine-5′-O-[α-naphthyl(benzyl-L-alaninyl)] phosphate (**1**)*

To a solution of 5-fluoro-2′-deoxyuridine (Carbosynth) (0.25 g, 1.01 mmol) in dry THF (10 mL) at 0 °C under argon atmosphere, NMI (0.40 mL, 5.07 mmol) was added dropwise. The reaction mixture was allowed to stir for 30 min, and then a solution of 1-naphthyl-(benzyl-L-alaninyl) phosphorochloridate (**6**) (0.82 g, 3.04 mmol) dissolved in anhydrous THF (3 mL) was added dropwise. The reaction mixture was stirred at room temperature for 16 h and then evaporated *in vacuo* to give a residue that was re-dissolved in CH_2Cl_2 (Fisher Scientific), washed twice with 0.5 M HCl (Sigma–Aldrich) (2 × 5 mL). The organic phase was purified by column chromatography on silica gel, eluting with CH_2Cl_2–MeOH (Fisher Scientific) as a gradient (0–5% MeOH) to afford the product **1** as colorless solid (47.0 mg, 8%) [R_f = 0.19 (CH_2Cl_2–MeOH, 95:5)], (Found: MNa^+, 636.1520. $C_{29}H_{29}N_3O_9FNaP$ requires [MNa^+], 636.1523); ^{31}P NMR (202 MHz, MeOD, mixture of diastereoisomers 43%:57%): δ_P 4.61, 4.25; ^{19}F NMR (470 MHz, MeOD): δ_F −167.45, −167.25; ^1H NMR (500 MHz, MeOD): δ_H 8.18–8.12 (m, 1H, ArH), 7.90–7.86 (m, 1H, ArH), 7.72–7.67 (m, 2H, ArH, H-6), 7.55–7.47 (m, 3H, ArH), 7.45–7.27 (m, 6H, ArH), 6.16–6.06 (m, 1H, H-1′), 5.13, 5.08 (2 × AB system, 2H, J = 12.0 Hz, OCH_2Ph), 4.36–4.24 (m, 3H, 2 × H-5′, H-3′), 4.15–4.03 (m, 2H, $CHCH_3$, H-4′), 2.17-2.08 (m, 1H, H-2′), 1.79–1.67 (m, 1H, H-2′), 1.38–1.34 (m, 3H, $CHCH_3$); ^{13}C NMR (125 MHz, MeOD): δ_C 174.9 (d, $^3J_{C\text{-}P}$ = 4.3 Hz, C=O, ester), 174.6 (d, $^3J_{C\text{-}P}$ = 5.0 Hz, C=O, ester), 159.3 (d, $^2J_{C\text{-}F}$ = 26.1 Hz, CH-base), 150.5 (d, $^4J_{C\text{-}F}$ = 4.0 Hz, C=O, base), 147.9 (d, $^2J_{C\text{-}P}$ = 7.4 Hz, C-Ar, Naph), 147.8 (d, $^2J_{C\text{-}P}$ = 7.7 Hz, C-Ar, Naph),



Scheme 4. *Reagents and conditions*: (i) *t*BuMgCl, dry THF, then phosphorochloridate (**6**), R.T., 16 h.

141.7 (d, $^{1}J_{C\text{-}F}$ = 233.9 Hz, *C*F), 141.6 (d, $^{1}J_{C\text{-}F}$ = 233.8 Hz, *C*F), 137.2, 137.1, 136.2 (*C*-Ar), 129.7, 129.6, 129.5, 129.4, 129.0, 128.9, 128.1, 128.0 (*C*H-Ar), 127.9, 127.8 (*C*-Ar), 127.7, 127.6, 126.6, 126.5, 126.2, 125.7, 125.6, 125.4, 125.3, 122.6 (*C*H-Ar), 116.5 (d, $^{3}J_{C\text{-}P}$ = 3.5 Hz, *C*H-Ar), 116.2 (d, $^{3}J_{C\text{-}P}$ = 3.3 Hz, *C*H-Ar), 87.0, 86.9 (*C*-1′), 86.8, 86.7 (2 × d, $^{3}J_{C\text{-}P}$ = 8.1 Hz, *C*-4′), 72.1, 72.0 (*C*-3′), 68.1, 68.0 (*C*H$_2$Ph), 67.8, 67.6 (2 × d, $^{2}J_{C\text{-}P}$ = 5.2 Hz, *C*-5′), 51.9, 51.8 (*C*HCH$_3$), 40.9, 40.8 (*C*-2′), 20.5 (d, $^{3}J_{C\text{-}P}$ = 6.5 Hz, CHCH$_3$), 20.3 (d, $^{3}J_{C\text{-}P}$ = 7.6 Hz, CHCH$_3$); *m*/*z* (ES) 636 (MNa$^+$, 100%), reverse HPLC eluting with (H$_2$O/MeOH from 100/0 to 0/100) in 45 min, showed two peaks of the diastereoisomers with t_R 34.23 min and t_R 34.59 min. Analytically calculated for $C_{29}H_{29}FN_3O_9P$: C, 56.77; H, 4.76; N, 6.85. Found: C, 56.57; H, 5.06; N, 6.72; UV (0.05 M phosphate buffer (Sigma–Aldrich), pH 7.4) λ_{max} = 271 nm (ε_{max} = 7050); log P (octanol (Sigma–Aldrich):water) measured: 1.7, calculated 3.53 (Chemoffice 11.0).

2.1.5. Synthesis of 5-fluoro-2′-deoxyuridine-5′-*O*-[α-naphthyl(benzyl-L-alaninyl)] phosphate (1) – alternative method

To a solution of 5-fluoro-2′-deoxyuridine (0.35 g, 1.42 mmol) in dry THF (15 mL), *t*BuMgCl (1 M in THF, 0.40 mL, 1.56 mmol) was added dropwise. The reaction mixture was stirred for 30 min and then a solution of 1-naphthyl-(benzyl-L-alaninyl) phosphorochloridate (**6**) (1.14 g, 2.84 mmol) dissolved in anhydrous THF (3 mL) was added dropwise. The reaction mixture was stirred at room temperature for 16 h and then evaporated under reduced pressure to give a crude residue. Purification by column chromatography on silica gel (Merck Chemicals) eluting with gradient of MeOH (0–5%) in CH_2Cl_2 afforded the product **1** as a colorless solid (78 mg, 9%). (All the spectroscopic data of **1** are in agreement with data reported in Section 2.1.4.) Alongside with the desired product **1**, the 3′-ProTide analogue (**8**) was isolated and characterized by NMR and HPLC.

5-Fluoro-2′-deoxyuridine-3′-*O*-[α-naphthyl(benzyl-L-alaninyl)] phosphate (**8**) was obtained as a colorless solid (15.0 mg, 2%) [R_f = 0.50 (CH_2Cl_2–MeOH, 95:5)], (Found: MNa$^+$, 636.1525. $C_{29}H_{29}N_3O_9NaPF$ requires [MNa$^+$], 636.1523); ^{31}P NMR (202 MHz, MeOD): δ_P 3.79, 3.11; ^{19}F NMR (470 MHz, MeOD): δ_F −168.62, −168.53; ^{1}H NMR (500 MHz, MeOD): δ_H 8.24–8.15 (m, 2H, Ar*H*), 7.94–7.88 (m, 1H, Ar*H*), 7.73 (d, 1H, $^{3}J_{C\text{-}F}$ = 7.9 Hz, *H*-6), 7.60–7.40 (m, 3H, Ar*H*), 7.38–7.24 (m, 6H, Ar*H*), 6.28–6.17 (m, 1H, *H*-1′), 5.14–5.11 (m, 2H, *CH*$_2$Ph), 4.21–4.03 (m, 2H, *CH*CH$_3$, *H*-3′), 3.93–3.63 (m, 3H, 2 × *H*-5′, *H*-4′), 2.51–2.43 (m, 1H, *H*-2′), 2.32–2.23 (m, 1H, *H*-2′), 1.37 (d, 3H, *J* = 7.2 Hz, CHC*H*$_3$, one diast.), 1.35 (d, 3H, *J* = 7.2 Hz, CHC*H*$_3$, one diast.); ^{13}C NMR (125 MHz, MeOD): 174.6 (d, $^{3}J_{C\text{-}P}$ = 4.8 Hz, *C*=O ester), 174.4 (d, $^{3}J_{C\text{-}P}$ = 5.1 Hz, *C*=O ester), 159.1 (d, $^{2}J_{C\text{-}F}$ = 26.0 Hz, *C*-base), 150.2 (d, $^{4}J_{C\text{-}F}$ = 4.1 Hz, *C*=O, base), 148.0 (d, $^{2}J_{C\text{-}P}$ = 7.2 Hz, *C*-Ar, Naph), 147.9 (d, $^{2}J_{C\text{-}P}$ = 7.2 Hz, *C*-Ar, Naph), 141.4 (d, $^{1}J_{C\text{-}F}$ = 233.0 Hz, *C*F), 141.2 (d, $^{1}J_{C\text{-}F}$ = 233.0 Hz, *C*F), 137.5, 137.4, 136.3 (*C*-Ar), 130.8, 129.6, 129.5, 129.3, 129.2, 129.0, 128.9, 128.1, 128.0, 127.8, 127.6, 126.2, 126.1, 125.7, 122.6, 116.7, 116.3, 106.5 (*C*H-Ar), 87.5, 87.4 (*C*-1′), 86.6 (*C*-4′), 79.7 (d, $^{4}J_{C\text{-}P}$ = 4.9 Hz, *C*-3′), 68.0 (*C*-5′), 62.6 (*C*H$_2$Ph), 51.8 (*C*HCH$_3$), 39.9 (*C*-2′), 20.4, 20.3 (CHCH$_3$); *m*/*z* (ES) 613 (M$^+$, 100%); reverse-phase HPLC eluting with H$_2$O/MeOH from 100/0 to 0/100 in 45 min, 1 ml/min, l = 275 nm, showed two peaks of the diastereoisomers with t_R 26.69 min and t_R 27.41 min (23%:72%).

2.1.6. Synthesis of 5-fluoro-2′-deoxyuridine-5′-*O*-[(L-alaninyl)] phosphate ammonium salt (11)

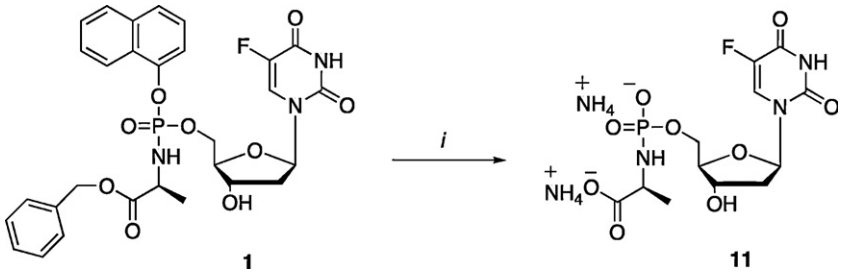
5-Fluoro-2′-deoxyuridine-5′-*O*-[(L-alaninyl)] phosphate ammonium salt was prepared by dissolving 5-fluoro-2′-deoxyuridine-5′-*O*-[α-naphthyl(benzyl-L-alaninyl)] phosphate (**1**) (0.08 g, 0.130 mmol) in a solution of triethylamine (5 mL) and water (5 mL) (Scheme 5). The reaction mixture was stirred at 35 °C for 16 h and then the solvents were removed under reduced pressure. The residue was treated with water and extracted with dichloromethane (Fisher Scientific). The aqueous layer was concentrated and evaporated under reduced pressure, then the resultant crude material was purified by column chromatography on silica, eluting with 2-propanol–H$_2$O–NH$_3$ (Fisher Scientific) (8:1:1) to afford the title compound **11** as a colorless solid (15.0 mg, 30%) [R_f = 0.04 (2-propanol–H$_2$O–NH$_3$ (8:1:1))]; ^{31}P NMR (202 MHz, D$_2$O): δ_P 7.13; ^{19}F NMR (470 MHz, D$_2$O): δ_F −168.00; ^{1}H NMR (500 MHz, D$_2$O): δ_H 7.93 (d, 1H, $^{3}J_{H\text{-}F}$ = 6.1 Hz, *H*-6), 6.25–6.30 (m, 1H, *H*-1′), 4.44–4.49 (m, 1H, *H*-3′), 4.06–4.11 (m, 1H, *H*-4′), 3.94–3.83 (m, 2H, C*H*$_2$OPh), 3.53 (q, 1H, *J* = 7.5 Hz, C*H*CH$_3$), 2.28–2.37 (m, 2H, *H*-2′), 1.19–1.25 (m, 3H, CHC*H*$_3$); ^{13}C NMR (125 MHz, MeOD): δ_C 174.8 (d, $^{3}J_{C\text{-}P}$ = 4.6 Hz, *C*=O), 159.2 (d, $^{2}J_{C\text{-}F}$ = 26.2 Hz, *C*H-base), 150.3 (d, $^{4}J_{C\text{-}F}$ = 4.0 Hz, *C*=O, base), 141.8 (d, $^{1}J_{C\text{-}F}$ = 233.8 Hz, *C*F-base), 87.0 (*C*-1′), 86.7 (d, $^{3}J_{C\text{-}P}$ = 7.5 Hz, *C*-4′), 71.1 (*C*-3′), 67.2 (d, $^{2}J_{C\text{-}P}$ = 5.5 Hz, *C*-5′), 51.0 (*C*HCH$_3$), 40.2 (*C*-2′), 20.3 (d, $^{3}J_{C\text{-}P}$ = 7.2 Hz, CHCH$_3$); *m*/*z* (ES) 396.1 (M−2NH$_4^-$+H]$^-$, 100%).

2.2. Radioactive pyrimidine deoxynucleosides

[5-^{3}H]dCyd (radiospecificity: 22 Ci/mmol) and [5-^{3}H]dUrd (radiospecificity: 15.9 Ci/mmol) were obtained from Moravek Biochemicals Inc. (Brea, CA).

2.3. Cell cultures

Murine leukaemia L1210/0 and human T-lymphocyte CEM/0 cells were obtained from the American Type Culture Collection (ATCC) (Rockville, MD). Human glioblastoma U87 cells were kindly provided by Dr. E. Menue (Institut Pasteur, Paris, France). Thymidine kinase-deficient CEM/TK$^-$ cells were a kind gift from Prof. S. Eriksson (currently at Uppsala University, Uppsala, Sweden) and Prof. A. Karlsson (Karolinska Institute, Stockholm, Sweden). Thymidine kinase-deficient L1210/TK$^-$ were derived from L1210/0 cells after selection for resistance against 5-bromo-2′-dUrd [33]. Infection of relevant cell lines with *Mycoplasma hyorhinis* (ATCC) resulted in chronically infected cell lines further referred to as L1210.Hyor and U87.Hyor. All cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) with 10% foetal bovine serum (FBS) (Biochrom AG,



Scheme 5. *Reagents and conditions*: (i) Et$_3$N:H$_2$O (1:1), 35 °C, 16 h.

Berlin, Germany), 10 mM Hepes and 1 mM sodium pyruvate (Invitrogen). Cells were grown at 37 °C in a humidified incubator with a gas phase of 5% CO_2.

2.4. Cytostatic assays

Monolayer cells (U87 and U87.Hyor) were seeded in 48-well microtiter plates (Nunc™, Roskilde, Denmark) at 10 000 cells/well. After 24 h, an equal volume of fresh medium containing the test compounds was added. On day 5, cells were trypsinized and counted in a Coulter counter (Analis, Suarlée, Belgium). Suspension cells (L1210/0, L1210/TK$^-$, L1210.Hyor, CEM/0, CEM/TK$^-$) were seeded in 96-well microtiter plates (Nunc™) at 60 000 cells/well in the presence of a given amount of the test compounds. The cells were allowed to proliferate for 48 h (L1210) or 72 h (CEM) and were then counted in a Coulter counter. The 50% inhibitory concentration (IC_{50}) was defined as the compound concentration required to reduce the number of viable cells by 50%.

2.5. Exposure of 5-FdUrd and NUC-3073 to Escherichia coli-encoded TP and human-encoded TP and UP

The substrate specificity of TP towards natural thymidine (dThd) (Sigma–Aldrich), uridine (Urd) (Sigma–Aldrich), 5-FdUrd (Sigma–Aldrich) and NUC-3073 was investigated by high pressure liquid chromatography (HPLC) (Waters, Milford, MA). Reaction mixtures containing 100 μM test compound and recombinant TP or uridine phosphorylase (UP) (human TP: 8.6 ng/μL; *E. coli* TP: 3.0 ng/μL; human UP: 4.0 ng/μL) in a total volume of 500 μL reaction buffer (10 mM Tris–HCl (Sigma–Aldrich); NaCl (Sigma–Aldrich) (150 μM for TP and 300 μM for UP); 1 mM EDTA (Sigma–Aldrich); 2 mM KH_2PO_4 (Merck Chemicals Ltd)/K_2HPO_4 (Acros Organics, Geel, Belgium)) were incubated at room temperature. At different time points (i.e. 0, 20, 40 min) 100 μL aliquots of the reaction mixtures were withdrawn and heated at 95 °C for 3 min to inactivate the enzyme. The resulting reaction products were separated on a reverse-phase RP-8 column (Merck Chemicals Ltd) and quantified by HPLC analysis (Alliance 2690, Waters). The separation of dThd from thymine was performed by a linear gradient from 98% separation buffer (50 mM NaH_2PO_4 (Acros Organics) and 5 mM heptane sulfonic acid (Sigma–Aldrich), pH 3.2) and 2% acetonitrile (BioSolve BV, Valkenswaard, the Netherlands), to 20% separation buffer + 80% acetonitrile (8 min 98% separation buffer + 2% acetonitrile; 5 min linear gradient of 98% separation buffer + 2% acetonitrile to 20% separation buffer + 80% acetonitrile; 10 min 20% separation buffer + 80% acetonitrile, followed by equilibration at 98% separation buffer + 2% acetonitrile). UV-based detection was performed at 267 nm. The separation of Urd from uracil was performed by a linear gradient from 100% separation buffer (see above) to 60% separation buffer + 40% acetonitrile (3 min 100% separation buffer; 6 min linear gradient of 100% separation buffer to 60% separation buffer + 40% acetonitrile; 6 min 60% separation buffer + 40% acetonitrile, followed by equilibration at 100% separation buffer). UV-based detection was performed at 258 nm.

2.6. Thymidylate synthase (TS) activity measurements

The activity of TS in intact L1210/0 and L1210/TK$^-$ cells was measured by evaluation of tritium release from [5-^{3}H]dUMP (formed in the cells from [5-^{3}H]dUrd or [5-^{3}H]dCyd) in the reaction catalysed by TS. This method has been described in detail by Balzarini and De Clercq [34]. Briefly, cell cultures (500 μL DMEM culture medium) were prepared containing ~3×10^6 L1210 cells and appropriate amounts of the test compounds (5-FdUrd and NUC-3073). After 30 min, 2 h and 4 h pre-incubation of the cells with the compounds at 37 °C, 1 μCi of [5-^{3}H]dUrd or [5-^{3}H]dCyd was added to the cell cultures. After 30 min incubation, 100 μL of the cell suspensions were withdrawn and added to a cold suspension of 500 μL activated charcoal (VWR, Haasrode, Belgium) (100 mg/ml in TCA (Merck Chemicals Ltd) 5%). After 10 min, the suspension was centrifuged at 13 000 rpm for 10 min, after which the radioactivity in 400 μL supernatant was counted in a liquid scintillator using OptiPhase HiSafe (Perkin Elmer, Waltham, MA).

2.7. Stability assays

2.7.1. Carboxypeptidase Y (EC 3.4.16.1) assay

The enzymatic stability of the prodrug NUC-3073 towards carboxypeptidase Y (Sigma–Aldrich) was studied using *in situ* ^{31}P NMR (202 MHz). The experiment was carried out by dissolving NUC-3073 (3.0 mg) in d_6-acetone (Cambridge Isotope Laboratories, Andover, MA) (150 μL) and adding TRIZMA (Sigma–Aldrich) buffer pH 7.6 (300 μL). The resulting solution was placed in an NMR tube and a ^{31}P NMR experiment at 25 °C was recorded as the blank experiment. The enzyme carboxypeptidase Y (0.2 mg) was dissolved in TRIZMA (150 μL) and added to the solution of the phosphoramidate derivative in the NMR tube. Next, the tube was placed in the NMR machine, which was set to run a ^{31}P NMR experiment (64 scans) every 4 min for 14 h at 25 °C. Data were processed and analyzed with the Bruker Topspin 2.1 program.

2.7.2. Human serum

The stability of the prodrug NUC-3073 in the presence of human serum was studied using *in situ* ^{31}P NMR (202 MHz). The ProTide NUC-3073 (**1**) (5.0 mg) was dissolved in a mixture of DMSO (Sigma–Aldrich)/D_2O (Cambridge Isotope Laboratories) (0.05 mL/0.15 mL) and then subjected to ^{31}P NMR analysis at 37 °C. After the first spectrum (control) was recorded, the NMR sample was treated with human serum (Sigma–Aldrich) (0.3 mL) and immediately subjected to further ^{31}P NMR experiments at 37 °C. ^{31}P NMR data were recorded every 15 min over 12 h and 30 min. In order to improve the visualization of the results all the spectra were further processed using the Lorentz–Gauss deconvolution method. Data recorded were processed and analyzed with the Bruker TopSpin 2.1 programme.

2.7.3. Buffer at pH 1

The stability of the prodrug NUC-3073 towards hydrolysis at pH 1 was studied using *in situ* ^{31}P NMR (202 MHz). The ProTide NUC-3073 (**1**) (2.6 mg) was dissolved in MeOD (Cambridge Isotope Laboratories) (0.1 mL) after which 0.5 mL buffer (pH 1, prepared from equal parts of 0.2 M HCl and 0.2 M KCl (Sigma–Aldrich)) was added. Then, on this sample ^{31}P NMR experiments were carried out at 37 °C recording the data every 12 min for 14 h. Data were processed and analyzed with the Bruker TopSpin 2.1 programme.

2.7.4. Buffer at pH 8

The stability of the prodrug NUC-3073 towards hydrolysis at pH 8 was studied using *in situ* ^{31}P NMR (202 MHz). The ProTide NUC-3073 (**1**) (4.9 mg) was dissolved in MeOD (0.1 mL) after which 0.5 mL buffer (pH 8, prepared from a solution of 0.1 M Na_2HPO_4 (Sigma–Aldrich), which was adjusted by 0.1 M HCl) was added. Next, on this sample ^{31}P NMR experiments were carried out at 37 °C recording the data every 12 min for 14 h. Data were processed and analyzed with the Bruker TopSpin 2.1 programme.

Table 1
Cytostatic activity of 5-FdUrd and NUC-3073 as represented by the IC_{50} value in different cell lines.

Cell lines	IC_{50}[a] (μM)	
	5-FdUrd	NUC-3073
L1210/0	0.0008 ± 0.000095	0.011 ± 0.0065
L1210/TK⁻	3.1 ± 0.14	0.027 ± 0.0028
L1210.Hyor	0.24 ± 0.054	0.025 ± 0.0073
CEM/0	0.028 ± 0.0014	0.089 ± 0.030
CEM/TK⁻	1.5 ± 0.071	0.32 ± 0.049
U87	0.007 ± 0.001	0.035 ± 0.0005
U87.Hyor	3.0 ± 0.55	0.039 ± 0.0025

[a] 50% inhibitory concentration or compound concentration required to inhibit tumour cell proliferation by 50%.

3. Results

3.1. Cytostatic activity of 5-FdUrd and its prodrug NUC-3073 against TK-competent and TK-deficient tumour cell lines

The cytostatic activity of 5-FdUrd and NUC-3073 was determined in different TK-expressing and TK-deficient tumour cell lines. As shown in Table 1, 5-FdUrd is strongly dependent on the expression of TK for its cytostatic activity. Its IC_{50} increased 4000-fold for L1210/TK⁻ cells (IC_{50}: 3.1 μM) *versus* wild-type L1210/0 cells (IC_{50}: 0.0008 μM) and 50-fold for CEM/TK⁻ cells (IC_{50}: 1.5 μM) *versus* CEM/0 cells (IC_{50}: 0.028 μM). In contrast, the cytostatic activity of the 5-FdUrd prodrug NUC-3073 remained virtually unchanged in TK-deficient cells when compared with wild-type cells (IC_{50}: 0.027 and 0.011 μM for L1210/TK⁻ and L1210/0, and 0.32 and 0.089 μM for CEM/TK⁻ and CEM/0 cells, respectively). Although the cytostatic activity of NUC-3073 was 3–10-fold inferior to 5-FdUrd against wild-type L1210/0 and CEM/0 cells, it proved 5–100-fold superior to 5-FdUrd in the TK-deficient tumour cell lines (Table 1).

3.2. Effect of mycoplasma infection of tumour cell cultures on the cytostatic activity of 5-FdUrd and its prodrug NUC-3073

The L1210/0 cell cultures were infected with the mycoplasma species *M. hyorhinis* (cells designated: L1210.Hyor). 5-FdUrd lost its cytostatic activity against the mycoplasma-infected L1210.Hyor cells by 300-fold (IC_{50}: 0.24 μM). Also, 5-FdUrd lost its cytostatic activity by 400-fold in U87.Hyor cell cultures when compared with uninfected U87 cells (Table 1). In sharp contrast, the 5-FdUrd prodrug NUC-3073 kept a similar cytostatic potential in both L1210/0 and L1210.Hyor cell cultures (IC_{50}: 0.011 and 0.025 μM, respectively). A similar observation was made for this prodrug when evaluated for its cytostatic activity in U87 and U87.Hyor cell cultures (IC_{50}: 0.035 and 0.039 μM, respectively). Thus, whereas the free nucleoside 5-FdUrd markedly lost its cytostatic potential against *M. hyorhinis*-infected tumour cell lines, the antiproliferative potential of its prodrug NUC-3073 was independent of the mycoplasma infection.

3.3. Phosphorolysis of 5-FdUrd and NUC-3073 by thymidine and uridine phosphorylases

5-FdUrd and its prodrug NUC-3073 were exposed to purified TP derived from *E. coli* or human erythrocytes, and to purified UP derived from human tumour tissue. Whereas *E. coli* and human TP rapidly converted dThd and 5-FdUrd to their free bases, NUC-3073 was completely stable in the presence of these enzymes (Fig. 2, panels A and B). Under similar experimental conditions, uridine was converted to uracil by human UP, but not by *E. coli* TP, or human TP. When both compounds were exposed to UP, dThd and NUC-3073 were not affected by the enzyme, whereas 5-FdUrd was slightly hydrolyzed (Fig. 2, panel C).

3.4. Inhibition of thymidylate synthase (TS) by 5-FdUrd and NUC-3073

The major target for the cytostatic activity of 5-FdUrd is TS. The activity of TS in intact tumour cells can be directly monitored by measuring the tritium release in intact L1210/0 cell cultures that were exposed to [5-^{3}H]deoxyuridine ([5-^{3}H]dUrd) or [5-^{3}H]deoxycytidine ([5-^{3}H]dCyd). Indeed, after intracellular conversion of [5-^{3}H]dUrd or [5-^{3}H]dCyd to [5-^{3}H]dUMP, the C-5 tritium atom on the pyrimidine base is released during the TS-catalysed reductive methylation. The ability of 5-FdUrd and its prodrug NUC-3073 to inhibit tritium release from [5-^{3}H]dUrd and [5-^{3}H]dCyd was therefore evaluated in L1210/0 cell cultures at a variety of compound concentrations. 5-FdUrd proved to be a potent inhibitor of TS *in situ*. Its IC_{50} for tritium release from [5-^{3}H]dCyd and [5-^{3}H]dUrd was around 0.0007–0.0009 μM (Table 2). The inhibitory activity of NUC-3073 on tritium release was much less

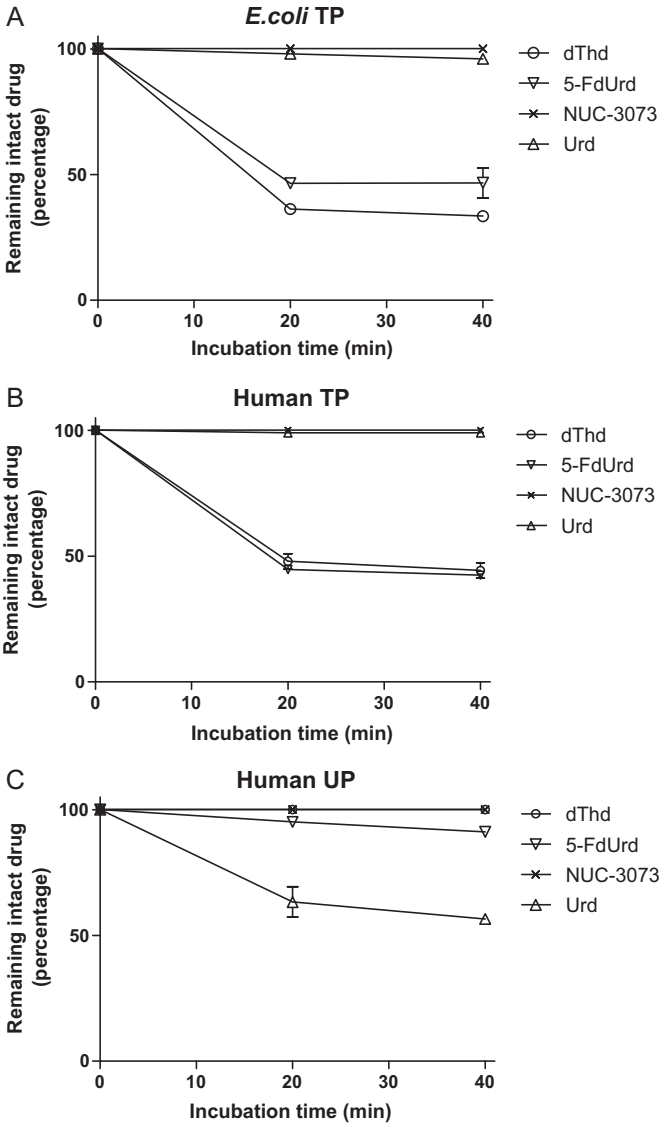


Fig. 2. Effect of thymidine phosphorylase from *E. coli* (panel A) and human (panel B) source and human uridine phosphorylase (panel C) on dThd, Urd, 5-FdUrd and NUC-3073. Data are the mean of at least 2 independent experiments (±S.D.).

Table 2
IC_{50} values of 5-FdUrd and NUC-3073 against TS in intact L1210/0 tumour cells (as determined by tritium release from [5-^{3}H]dUrd and [5-^{3}H]dCyd after 30 min exposure to the drugs).

Compound	IC_{50}[a] (μM)	
	Tritium release from [5-^{3}H]dUrd	Tritium release from [5-^{3}H]dCyd
5-FdUrd	0.0009 ± 0.0002	0.0007 ± 0.003
NUC-3073	0.16 ± 0.05	0.19 ± 0.08

[a] 50% inhibitory concentration or compound concentration required to inhibit tritium release from [5-^{3}H]dUrd or [5-^{3}H]dCyd in drug-exposed L1210/0 cell cultures by 50%.

(~200-fold) pronounced than that of 5-FdUrd, especially after only 30 min preincubation of the cells with the drugs (IC_{50}: 0.16–0.19 μM). However, longer pre-incubation times of the cells (up to 4 h) with 5-FdUrd and NUC-3073 before measuring TS activity in the intact tumour cells revealed a much more pronounced inhibitory activity of the prodrug against TS *in situ* (Fig. 3). Indeed, whereas the inhibition of ^{3}H release was only 2-fold increased upon longer pre-incubation times of 5-FdUrd, the inhibitory potential of NUC-3073 increased 10-fold (Fig. 3, panels A and B, and C and D). Interestingly, pre-incubation of the tumour cells with 5-FdUrd and NUC-3073 for at least 4 h results in TS inhibition in the intact tumour cells at drug concentrations that are very comparable with the 50% cytostatic activity concentrations of these drugs. Taken all data together, our observations indicate that the 5-FdUrd prodrug needs several metabolic

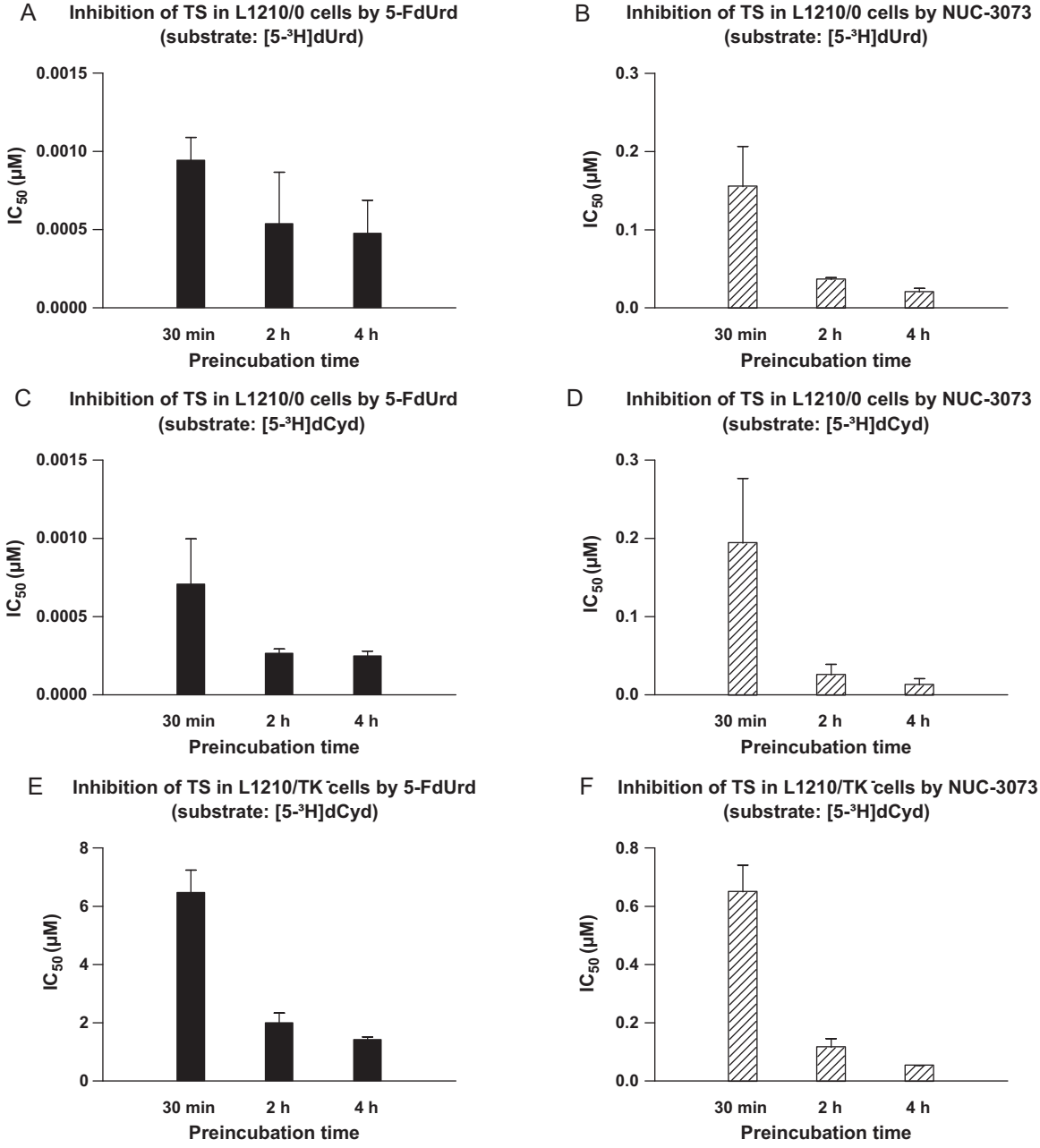


Fig. 3. Inhibition of TS by 5-FdUrd and NUC-3073 as measured by tritium release from [5-^{3}H]dUrd (panels A and B) and [5-^{3}H]dCyd (panels C and D) in L1210/0 cell cultures and by tritium release from [5-^{3}H]dCyd (panels E and F) in L1210/TK$^-$ cell cultures. Data are the mean of 2 independent experiments (±S.E.M.).

Table 3
IC_{50} values of 5-FdUrd and NUC-3073 against TS in intact L1210/0 and L1210/TK^- cells (as determined by tritium release from [5-^{3}H]dCyd after 4 h of preincubation with the products).

Compound	IC_{50}[a] (μM)	
	L1210/0	L1210/TK^-
5-FdUrd	0.0003 ± 0.00003	1.42 ± 0.09
NUC-3073	0.013 ± 0.008	0.053 ± 0.0009

[a] 50% inhibitory concentration or compound concentration required to inhibit tritium release from [5-^{3}H]dCyd in drug-exposed L1210 cells by 50% upon pre-exposure of the tumour cells for 4 h to 5-FdUrd or its prodrug NUC-3073.

conversion steps before reaching TS as the target enzyme for inhibition, and support the view that NUC-3073 acts as an efficient prodrug of 5-FdUrd to exert its eventual cytostatic activity.

The activity of TS in the presence of 5-FdUrd and NUC-3073 was also measured in intact L1210/TK^- cells using [5-^{3}H]dCyd as an externally supplied substrate (due to TK deficiency, [5-^{3}H]dUrd cannot be used). As demonstrated in Table 3 and Fig. 3 (panels E and F), the concentration of 5-FdUrd required to cause 50% inhibition of TS increased by a factor 5700 in TK-deficient L1210/TK^- cells (IC_{50}: 1.4 μM) when compared to wild-type L1210/0 cells (IC_{50}: 0.0003 μM). In contrast, the inhibitory activity of NUC-3073 against TS remained virtually unchanged in L1210/TK^- cells (IC_{50}: 0.053 μM in L1210/TK^- cells *versus* 0.013 μM in L1210/0 cells).

3.5. Metabolism of 5-FdUrd phosphoramidates

As shown in Fig. 4, the putative mechanism of activation of ProTides inside the cell, after uptake, involves a first enzymatic activation step mediated by a carboxypeptidase-type enzyme that hydrolyzes the ester of the aminoacyl moiety (step a, compound **9**). Next, a spontaneous cyclization followed by subsequent, spontaneous displacement of the aryl group (step b) and opening of the unstable ring mediated by water (step c) can give compounds **10** and **11**, respectively. The last step involves a hydrolysis of the P–N bond mediated by a phosphoramidase-type enzyme (step d) with release of the nucleoside monophosphate (**12**) in the intact cell [13,35].

To prove the proposed metabolic scheme for NUC-3073 (**1**) and to determine whether the ester motif of the 5-FdUrd phosphoramidate derivative is cleaved-off or not, we carried out an enzyme incubation experiment designed to mimic the first stages of the putative activation in the intact tumour cells. The compound **1** was incubated with carboxypeptidase Y (also known as cathepsin A) in TRIZMA buffer and its conversion was monitored by ^{31}P NMR.

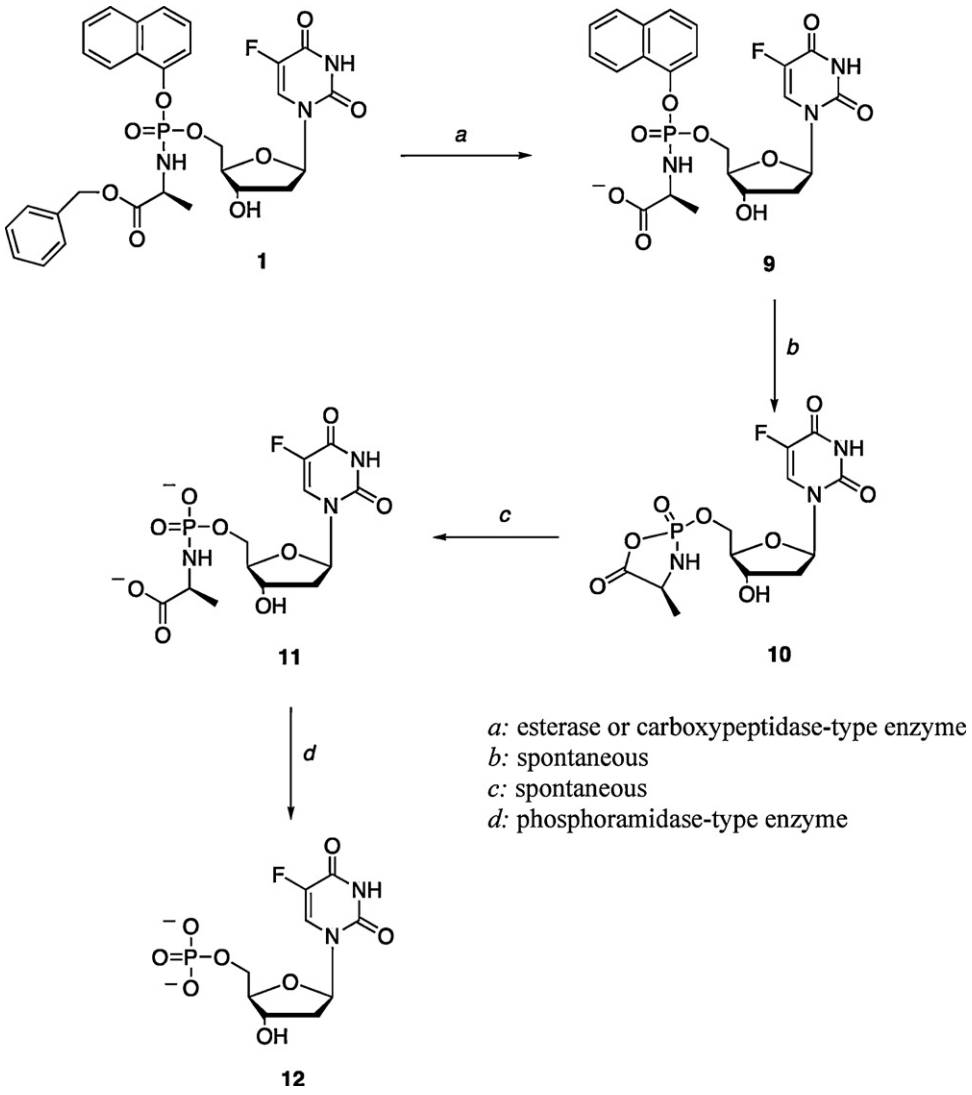


Fig. 4. Proposed putative mechanism of activation of the 5-FdUrd ProTide NUC-3073.

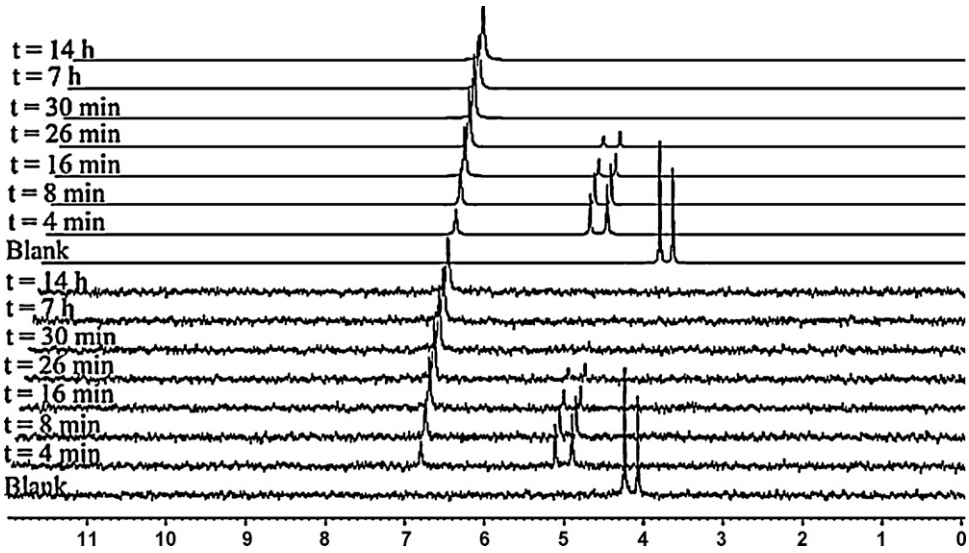


Fig. 5. Carboxypeptidase-mediated cleavage of prodrug NUC-3073 (**1**) monitored by ^{31}P NMR.

Spectra were recorded for 14 h acquiring scans at the periodic intervals every 4 min as shown in Fig. 5. For a better resolution original spectra (lower graphs) and deconvoluted ones (upper graphs) are shown.

Two peaks at δ 4.07; 4.23 ppm in the ^{31}P NMR spectrum correspond to two diastereoisomers of the parent compound **1**. Upon the addition of cathepsin A, during the first 4 min of the experiment the compound **1** was hydrolyzed to the corresponding intermediate(s) **9** (δ 4.95; 5.16 ppm), which lack the ester motif (Fig. 4). These intermediates did not persist as they were quickly converted to the aminoacyl phosphoramidate derivative (the final product in this assay) via the loss of the aryl group (steps a to c in Fig. 4). Due to the achirality at the phosphate the metabolite **11** appeared as a singlet (δ 6.85 ppm). Thus, the enzymatic assay spectra showed a fast metabolism of the parent $\sim\delta$ 4.00 with complete conversion to the putative intermediate within 26 min, which further stayed consistently present throughout the 14 h of the assay. The cleavage of the P–N bond releasing the nucleoside monophosphate was not detected in the enzyme experiment, as expected. This experiment indicates that the first activation step of ProTide NUC-3073 (**1**) may be sufficiently efficient, and therefore, may allow the eventual delivery of the nucleoside monophosphate metabolite in the intact tumour cells, following subsequent phosphoramidase-mediated cleavage of the amino acid to liberate FdUMP.

In order to support both, the putative mechanism proposed as well as the results obtained in the enzymatic assay, we decided to prepare the intermediate **11** via a synthetic route. Thus chemical hydrolysis of the compound **1** in the presence of triethylamine and water was performed (Scheme 5). The product **11** obtained as a diammonium salt was next added to the assay's final sample (containing only the enzymatic metabolite **11** in TRIZMA), and ^{31}P NMR analysis was investigated. The ^{31}P NMR spectrum has shown exclusively one peak at 6.85 ppm (data not shown); therefore our results have proved the proposed metabolic pathway and activation of ProTides.

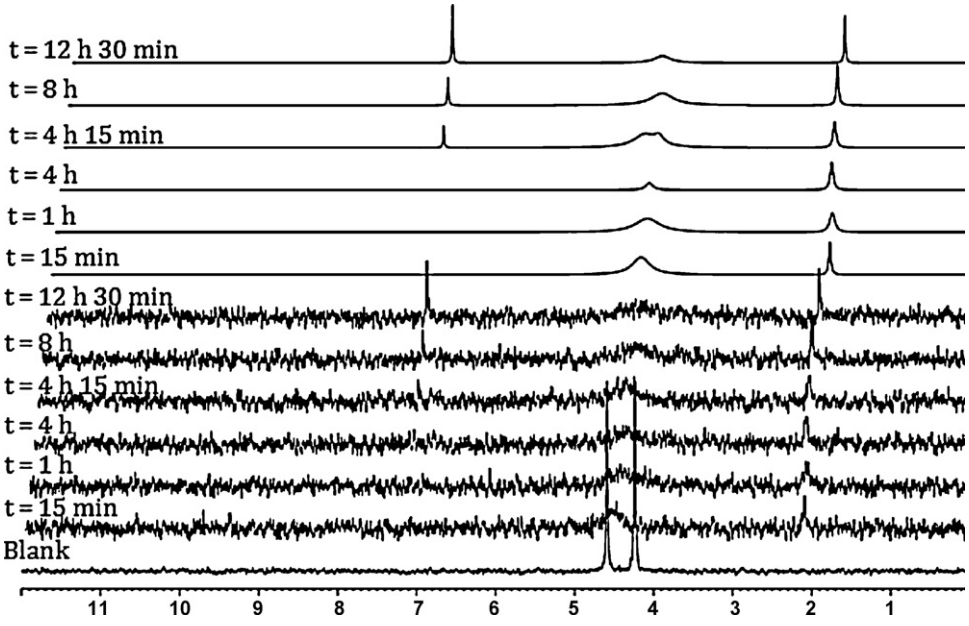


Fig. 6. ^{31}P NMR spectrum of compound NUC-3073 (**1**) in human serum.

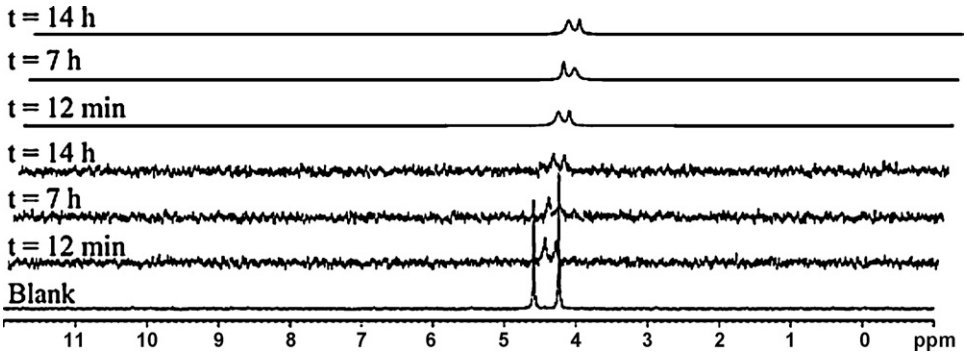


Fig. 7. ^{31}P NMR spectrum of compound NUC-3073 (**1**) in buffer pH 1.

3.6. Stability studies

In the current work also chemical stability studies on the prodrug NUC-3073 (**1**) have been performed by exposing the compound to human serum and to aqueous buffers (pH 1.0 and 8.0) using *in situ* ^{31}P NMR. Each experiment has been carried out dissolving the ProTide in the suitable deuterated solvent and analyzing the samples at 37 °C for about 14 h, acquiring scans at the regular time intervals. For a better resolution original spectra (lower graphs) and deconvoluted ones (upper graphs) are reported. The stability assay of the phosphoramidate NUC-3073 (**1**), after incubation in human serum, showed 73% of unchanged compound after 12 h and 30 min as shown in Fig. 6.

The spectra displayed a single peak inherent to the human serum at ~δ 2.00 and the double peak of the parent at ~δ 4.50 which after 4 h and 15 min was partly hydrolyzed to the aminoacyl phosphoramidate intermediate shown as a single peak at δ 7.20.

When chemical hydrolysis was evaluated at extreme experimental conditions, i.e. at pH 1.0 and pH 8.0 at 37 °C, a full stability of prodrug NUC-3073 (**1**) in both acidic and basic buffer conditions was observed. Spectra were recorded for 14 h acquiring scans every 12 min at regular intervals as shown in Figs. 7 and 8. The ProTide (**1**) examined at pH 1.0 showed constant two peaks of diastereoisomers at δ 4.35; 4.50 throughout the time of the assay (Fig. 7).

Also, at pH 8.0 the spectra displayed a persistent peak of the prodrug (**1**) at δ 4.48 and a single peak at δ 2.55 corresponding to a buffer peak (Fig. 8).

4. Discussion

Various mechanisms of tumour cell resistance towards fluoropyrimidines such as 5-FU, 5-FdUrd and trifluorothymidine (TFT) have been described, including a decreased activity of crucial drug-activating enzymes (e.g. TK and orotate phosphoribosyltransferase), an increased activity of drug-inactivating enzymes (i.e. TP), an upregulation of the target enzyme (e.g. TS) and/or defective facilitated diffusion of nucleosides [36–40]. Also, high TP levels found in several types of cancer tissue were reported to be predictive for a poorer prognosis upon treatment with fluoropyrimidines [41–43], although other studies have not confirmed these findings [43,44]. The study presented here was aimed at the development of a prodrug for 5-FdUrd, to circumvent possible resistance mechanisms and susceptibility to degradation by catabolic enzymes, present in the tumour micro-environment.

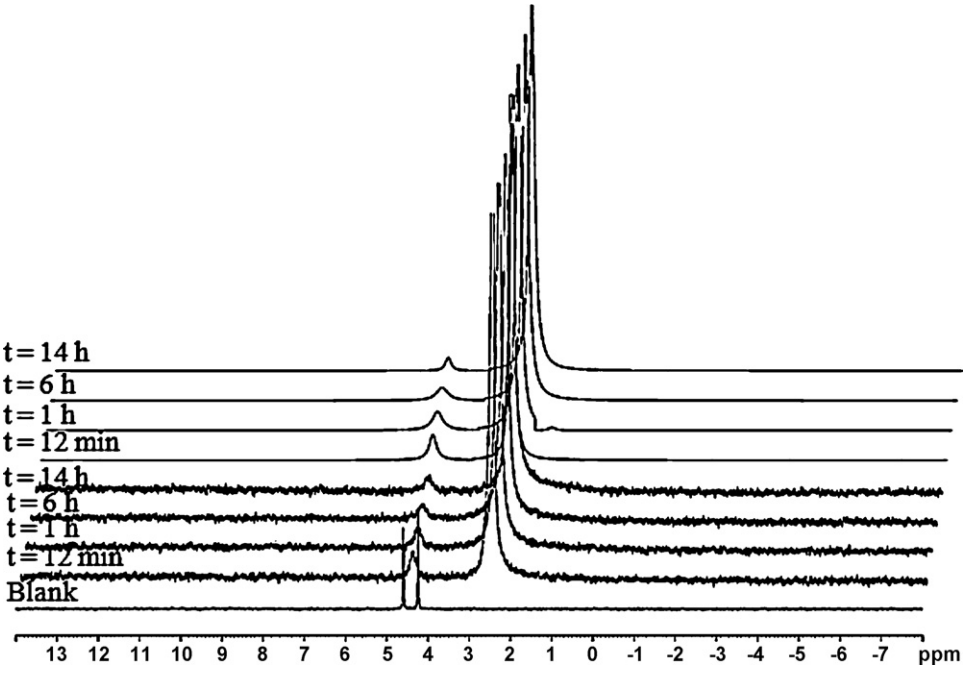


Fig. 8. ^{31}P NMR spectrum of compound NUC-3073 (**1**) in buffer pH 8.

We describe herein NUC-3073, a phosphoramidate prodrug of 5-FdUrd that may fulfil these aims. After uptake into the tumour cells, NUC-3073 generates 5-FdUMP intracellularly upon enzymatic cleavage. Our stability studies and enzyme/serum studies by ^{31}P NMR technology revealed that the prodrug NUC-3073 is fully stable in acid and alkaline conditions, but subject to hydrolysis in the presence of carboxypeptidase Y, or to some extent by serum resulting in the formation of the nucleoside 5′-phosphoramidate derivative. Whereas TK is a key enzyme in the activation of 5-FdUrd, NUC-3073 was found to be much less dependent on TK to exert its cytostatic action in both murine (L1210) and human (CEM) cell cultures. Due to the lipophilic nature of ProTides, these molecules can deliver nucleoside-monophosphates directly into the intact tumour cell after conversion to their nucleoside phosphoramidate derivative by enzymes such as carboxyesterases or carboxypeptidases (i.e. carboxypeptidase Y), eliminating the need for an initial phosphorylation by specific nucleoside kinases such as cytosolic TK-1. In this regard, NUC-3073 may be an adequate tool for the treatment of tumour cells with a modified TK-1 activity (either acquired or inherent). Also, since TK-1 expression is S-phase-dependent, it is expected that NUC-3073 can also efficiently deliver 5-FdUMP in tumour cells that are not in the S-phase of their replication cycle. Our TS activity studies revealed that NUC-3073 was able to inhibit TS in both wild-type and TK-deficient tumour cell lines, pointing again to an efficient intracellular delivery of the 5′-monophosphate of 5-FdUrd, and its virtual independence of cellular TK-1 for metabolic activation.

Normal tissue toxicity is always a concern in the development of anticancer drugs and in particular it might be a potential concern whether the 5-FdUrd prodrug may show an increased toxicity in non-proliferating cells or other cell types with low TK-1 activity. In such cell tissues, thymidine nucleotide pools are rather low, and thus, the released 5-FdUMP derivative and the di- and triphosphate drug metabolites may have a competitive advantage and exert a more pronounced cytotoxic activity. However, it should be kept in mind that in non-proliferating cells, TS activity is also markedly decreased due to lack of ongoing DNA synthesis. Thus, due to a lower degree of target enzyme (TS) activity in resting cells, it is expected that the released 5-FdUMP should not necessarily afford higher cell toxicity in such cells. It was indeed found that both 5-FdUrd and the 5-FdUrd prodrug NUC-3073 were markedly less toxic in confluent human cervix carcinoma HeLa/0 cell cultures compared with the toxicity values observed for proliferating L1210 and CEM cell cultures [minimal cytotoxic concentration (MCC) of NUC-3073 for HeLa: 2 μM]. Also, NUC-3073 was not found more toxic in confluent HeLa/TK$^-$ cells than in wild-type HeLa/0 cells (data not shown). Thus, evidence for increased toxicity of a prodrug such as NUC-3073 against non-proliferating tissue was not observed.

We also proved that NUC-3073 is unlikely to be inactivated by catabolic enzymes involved in nucleoside metabolism. Indeed, whereas 5-FdUrd is highly susceptible to enzymatic hydrolysis by TP resulting in the formation of 5-FU and 2′-deoxyribose-1-phosphate, we found that its prodrug NUC-3073 is not a substrate for prokaryotic (i.e. *E. coli*) or mammalian (i.e. human erythrocyte) TP. Also, UP (derived from human tumour tissue) does not recognize NUC-3073 as a substrate, whereas 5-FdUrd is (poorly, but measurably) hydrolyzed by this enzyme. Several studies revealed that many tumour cells have elevated levels of TP, which also acts as an angiogenic factor [43,45]. Moreover, there are several reports on the preferential colonization of tumour tissue by mycoplasmas [15–17] which interfere with the cytostatic activity of several conventional chemotherapeutics *in vitro* through its encoded TP [27–29]. Our findings that 5-FdUrd, but not NUC-3073, markedly loses cytostatic activity when the tumour cells are infected by (TP-expressing) mycoplasmas, is in full agreement with these observations. Therefore, the administration of a TP-insensitive anti-cancer prodrug such as NUC-3073, demonstrated to be chemically stable at extreme pH conditions, may further improve cancer chemotherapy.

It should be mentioned that the isopropylalaninyl monoamidate phenyl monoester prodrug of tenofovir (GS 7340) has been investigated for its pharmacokinetics in dogs [46]. This compound, being rather similar to NUC-3073 in terms of the prodrug moiety, had a half-life of 90 min in human plasma, and reached an oral bioavailability of ~17% after a single oral dose of GS 7340 (10 mg/kg) to beagle dogs. There was an increased distribution of the parent drug to tissues of lymphatic origin compared to the commercially available prodrug tenofovir-DF (Viread) [46]. No increased side-effects for GS 7340 were reported when compared with the clinically used Viread. These observations revealed that there may be a potential clinical application for prodrugs such as NUC-3073.

In conclusion, we believe that ProTides, such as NUC-3073, may represent an interesting new approach towards the development of more resilient anti-cancer drugs. NUC-3073 may have at least several advantages over its parent drug 5-FdUrd: it exerts its cytostatic activity independently of TK and it is resistant to metabolic breakdown by TP, an enzyme that is often upregulated in tumours or may be externally expressed by mycoplasma infection of the tumour tissue.

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