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Synthesis, anti-HIV and cytostatic evaluation of 3′-deoxy-3′-fluorothymidine (FLT) pro-nucleotides

Winnie Velanguparackel [a,†], Nadège Hamon [a,†], Jan Balzarini [b], Christopher McGuigan [a], Andrew D. Westwell [a,*]

[a] School of Pharmacy and Pharmaceutical Sciences, Cardiff University, Redwood Building, King Edward VII Avenue, Cardiff, CF10 3NB Wales, UK
[b] Rega Institute for Medical Research, KU Leuven, Minderbroedersstraat 10, B-3000 Leuven, Belgium

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ABSTRACT

A series of pro-nucleotide phosphoramidates and phosphorodiamidates of the antiviral lead compound 3′-deoxy-3′-fluorothymidine (FLT) have been designed and synthesized. In vitro antiretroviral and cytostatic studies revealed potent (sub-micromolar) inhibition of HIV-1 and HIV-2 replication, with retention of activity in thymidine kinase-negative cell models, as predicted by the ProTide concept.

Despite their crucial and widespread role as approved therapeutics for antiviral[1] and anticancer[2] therapy, the activity of nucleoside analogues is compromised by their requirement for active transporter-mediated cell uptake and bioactivation, usually via three successive phosphorylation steps. Since initial nucleoside kinase-catalysed phosphorylation is frequently the rate-limiting step in nucleoside drug activation, a number of pro-drug (pro-nucleotide) monophosphate strategies have been developed to circumvent delivery, uptake and metabolic activation issues.

One of the most widely used pro-nucleotide strategies is the approach pioneered by McGuigan,[3,4] in which the monophosphate is masked as a phosphoramidate,[5] or in more recent modalities as a phosphorodiamidate.[6] Following passive diffusion through the cell membrane, the pro-nucleotide (ProTide) breaks down within the cell via a well-studied mechanism to release the nucleoside monophosphate, trapped intracellularly and primed for further phosphorylation. The ProTide approach has been applied to improve efficacy for a wide range of antiviral and anticancer nucleosides. Recent highlights include the clinical trial candidates INX-189 (hepatitis C virus, to Phase 2)[7] and a pro-nucleotide of the cancer chemotherapeutic gemcitabine (NUC-1031, Phase 1, ongoing),[8] shown in Figure 1.

Fluorine is well known to impart rather special physicochemical and pharmacological properties on drug candidates, and is present in 15–20% of currently approved drugs overall.[9] Several fluorinated nucleosides have been applied as both therapeutic agents and in diagnostic imaging. Examples of fluorinated nucleoside antivirals include emtricitabine (HIV),[10] clevudine (HBV)[11] and trifluridine (HSV; cancer).[12,13] Besides trifluridine, fluorinated nucleoside-based anticancer drugs include also the 5-fluorouracil pro-drug such as capecitabine[14] and gemcitabine;[15] and the related purine nucleosides fludarabine and clofarabine.[16]

3′-Deoxy-3′-fluorothymidine (FLT) is a particularly interesting fluorinated nucleoside derivative possessing a variety of biological properties. The major clinical application of FLT is in the non-invasive diagnostic imaging of tumour proliferation using Positron Emission Tomography (PET), where the ^{18}F radiolabelled analogue (^{18}FLT, $t_{1/2}$ = 110 min.) is routinely employed as a highly sensitive imaging probe.[17] In terms of therapeutic application, FLT (also known as alovudine) has been shown to be a more potent inhibitor of HIV replication than the well-known anti-retroviral agent AZT. In vitro studies have shown that FLT inhibits replication of nucleoside analogue reverse transcriptase inhibitor (NRTI)-resistant HIV strains.[18] Further clinical development of FLT was halted, however, due to toxicological safety concerns.[19] Previous preliminary work

* Corresponding author. Tel.: +44 (0)2920 875800; fax: +44 (0)2920 874149.
E-mail address: WestwellA@cf.ac.uk (A.D. Westwell).
† Denotes equal contributions to the experimental work.

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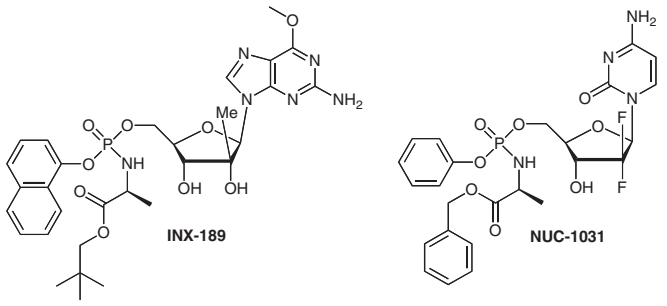


Figure 1. Chemical structures of ProTides INX-189 and NUC-1031.

on FLT pro-nucleotides as HIV inhibitors suggested the possibility to retain the potent activity of the parent compound using a ProTide-based strategy.[20] In this Letter, we describe the synthesis and in vitro evaluation of both phosphoramidate (**3a**–**m**) and phosphorodiamidate (**5a**–**b**) pro-nucleotide derivatives of FLT as antiretroviral and anticancer agents. These studies test the hypothesis that application of the ProTide delivery concept might generate highly potent new nucleotide analogues that are able to by-pass the requirement for a functional thymidine kinase (TK).

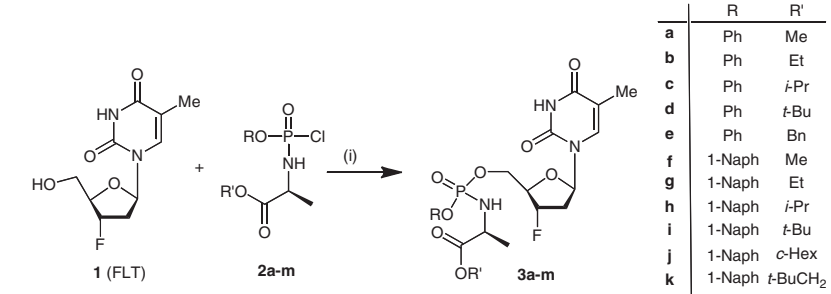
Synthesis of the FLT phosphoramidates was accomplished through application of known pro-nucleotide chemistry.[5] Reaction of the commercially available FLT (**1**, Carbosynth U.K.) with a range of previously reported aryloxyphosphorochloridates (**2a**–**m**), promoted by *tert*-butylmagnesium chloride as a hindered base in THF, provided the target phosphoramidate products in low to moderate isolated yield (high yield based on recovered starting material, BORSM) following column chromatography (Scheme 1).[21,22]

We then tried to synthesize diamidate prodrugs of FLT according to the trimethyl phosphate method used previously in our laboratory.[23] Unfortunately, after 5 h of reaction at −78 °C in the presence of TMP and $POCl_3$, the signal of the desired intermediate **4** on ^{31}P NMR was barely visible. After a further 15 h in the presence of L-Ala neopentyl ester and DIPEA, no signal of the desired diamidate prodrug was observed on ^{31}P NMR. We can explain this by a lack of solubility of FLT in the organic solvents used. Eventually symmetrical FLT phosphorodiamidates (**5a**–**b**) were synthesized according to literature methods[6,24] by treating FLT with phosphoryl chloride under basic conditions to generate an intermediate dichloridate (**4**) which was not isolated. Reaction of FLT dichloridate with further triethylamine, followed by excess α-amino acid ester, gave the required FLT phosphorodiamidates after purification by column chromatography and preparative TLC (Scheme 1).[25,26]

The resulting FLT Pro-Tides were evaluated for their in vitro inhibitory effect on the replication of HIV-1 and HIV-2 in human T-lymphocyte (CEM) cell cultures, according to previously described methods.[24,27] Importantly, TK^- mutant CEM cells were included to examine the hypothesis that the ProTide derivatives, unlike the parent compound FLT, would be able to by-pass the requirement for TK-directed phosphorylation and would be active in this test. The results of the HIV assays are shown in Table 1. FLT (**1**) was used as a positive control, confirming potent anti-HIV-1 and -HIV-2 activity in the TK-expressing CEM cells (EC_{50} = 6.2 and 18 nM, respectively), but not in the TK knockout CEM mutant cells (EC_{50} >50 μM). The >1000 fold loss of activity of (**1**) in the TK^- assay is notable.

Examination of the in vitro antiretroviral activity indicates some interesting and novel findings. Although the new pro-nucleotides were not generally found to be as active as FLT in TK-positive cells (CEM/0), some phosphoramidates (e.g., **3j**–**k**) and phosphorodiamidates (e.g., **5a**–**b**) gave EC_{50} values in the middle (⩽100 nM) nanomolar concentration range for HIV-1 and/or HIV-2. Phosphorodiamidate (**5b**) (R = neopentyl) was found to be the most potent compound in the assays. Importantly, the test compounds were able to retain moderate activity in the CEM/TK^- (TK mutant) cell cultures in the lower micromolar range, unlike FLT (with the exception of ProTides **3d**, **3h**–**i**, **3m** and **5b**). Based on the overall profile across these three test assays, FLT phosphoramidate (**3k**, R = naphthyl; R′ = neopentyl) and FLT phosphorodiamidate (**5a**, R = Bn) are the most potent and promising compounds for further study. Importantly, compound (**3k**) shows only a 10-fold loss of activity in the TK knockout CEM mutant cell assay, versus >1000-fold for compound (**1**).

In vitro examination of the cytostatic potential of the FLT ProTides was studied in the human T-lymphocyte CEM cells (both wild-type TK and TK-deficient (TK^-)), and the murine leukaemia cell line L1210, according to previously reported protocols.[24,28] The results of these studies are shown in Table 2.



	R	R'
a	Ph	Me
b	Ph	Et
c	Ph	*i*-Pr
d	Ph	*t*-Bu
e	Ph	Bn
f	1-Naph	Me
g	1-Naph	Et
h	1-Naph	*i*-Pr
i	1-Naph	*t*-Bu
j	1-Naph	*c*-Hex
k	1-Naph	*t*-BuCH$_2$
l	1-Naph	Bn
m	Me	Bn

Scheme 1. Synthesis of FLT pro-nucleotides.

Table 1
Anti-HIV-1 and -HIV-2 activity (EC_{50}, μM) values in human T-lymphocyte (CEM) cell cultures. Results are expressed as mean values of at least 2–3 independent experiments

Compd	EC_{50}/μM (CEM/0)		EC_{50}/μM (CEM/TK$^-$)
	HIV-1	HIV-2	HIV-2
3a	0.55 ± 0.21	1.3 ± 0.14	4.7 ± 4.8
3b	0.10 ± 0.068	0.24 ± 0.23	1.3 ± 0.71
3c	0.75 ± 0.0	1.1 ± 0.23	28 ± 2.1
3d	0.29 ± 0.16	0.90 ± 0.71	>50
3e	0.13 ± 0.11	0.18 ± 0.049	2.0 ± 0.0
3f	0.43 ± 0.35	0.65 ± 0.35	3.0 ± 1.3
3g	0.16 ± 0.028	0.18 ± 0.071	5.2 ± 0.92
3h	0.56 ± 0.23	1.0 ± 0.27	>50
3i	2.3 ± 1.4	4.7 ± 2.4	>50
3j	0.048 ± 0.078	0.14 ± 0.0071	2.0 ± 1.1
3k	0.032 ± 0.0057	0.080 ± 0.0	0.87 ± 0.33
3l	0.12 ± 0.014	0.18 ± 0.078	2.1 ± 1.2
3m	0.65 ± 0.21	1.6 ± 0.38	89 ± 8.5
5a	0.036 ± 0.0042	0.13 ± 0.0071	1.0 ± 0.87
5b	0.017 ± 0.0092	0.045 ± 0.012	20 ± 0.71
1	0.0062 ± 0.0021	0.018 ± 0.014	>50

Table 2
Cytostatic activity (IC_{50}, μM) values in human T-lymphocyte (CEM) and murine leukaemia (L1210) cells. Results are expressed as mean values of at least 2–3 independent experiments

Compd	IC_{50}/μM		
	CEM/0	CEM/TK$^-$	L1210
3a	>250	>250	8.2 ± 3.7
3b	>250	>250	4.4 ± 1.4
3c	145 ± 38	>250	23 ± 1
3d	124 ± 58	>250	6.3 ± 0.6
3e	117 ± 88	172 ± 59	5.8 ± 0.6
3f	66 ± 10	112 ± 52	3.0 ± 1.2
3g	81 ± 1	138 ± 5	6.0 ± 0.9
3h	206 ± 62	>250	24 ± 2
3i	148 ± 17	>250	36 ± 3
3j	13 ± 2	20 ± 3	2.9 ± 1.2
3k	14 ± 1	20 ± 3	1.9 ± 1.1
3l	54 ± 5	57 ± 8	5.2 ± 3.3
3m	100 ± 12	>250	43 ± 13
5a	17 ± 6	22 ± 5	1.2 ± 1.1
5b	66 ± 11	43 ± 34	3.8 ± 2.7
1	39 ± 26	>250	0.081 ± 0.037

Examination of Table 2 reveals few compounds with significantly potent cytostatic activity within these cell line models. The most potent activity was observed in the L1210/0 leukaemia cell line, where low micromolar IC_{50} values was found for a variety of compounds, but where cytostatic activity was lower than for the parental FLT (**1**). Gratifyingly, compounds with moderate activity in the CEM/0 cell line (such as **3j**, **3k** and **5a**) were largely able to retain activity within the CEM/TK$^-$ tumour cell model (unlike the parent FLT) and were non-toxic at antiviral concentrations.

In conclusion, the preparation of a series of phosphoramidate and phosphorodiamidate ProTides of the antiviral lead compound FLT have been prepared. In vitro screening with anti-HIV and anticancer model systems revealed compounds with generally lower activity than the parent lead, but with the ability to retain activity within TK$^-$ models. This retention of activity across the test panels provides further evidence for the potential of ProTides to, at least in part, by-pass the need of thymidine kinase-catalysed phosphorylation and deliver the intact nucleoside monophosphate ready for further metabolic conversion to the biologically active form.

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Supplementary data

Supplementary data (^{1}H, ^{13}C, ^{19}F and ^{31}P NMR, mass spectrometry and HPLC data for FLT pro-nucleotide analogues) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2014.03.092.

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21. *General method for synthesis of FLT phosphoramidates (**3a**–**m**).* A solution of *tert*-butylmagnesium chloride (1 M in THF, 1.3 equiv) was added dropwise to a stirring solution of 3′-deoxy-3′-fluorothymidine (FLT, **1**, 0.1 M) in anhydrous THF at room temperature, followed by stirring for 30 min. A solution of phosphochloridate (**2a**–**m**, 2.05 equiv) in anhydrous THF (1 mL) was then added dropwise to the reaction mixture followed by stirring for 14–22 h. THF was then removed in vacuo and the residue purified by column chromatography ($CHCl_3$/MeOH 100/0 to 95/5) followed by preparative TLC ($CHCl_3$/MeOH 95/5) to give the required FLT phosphoramidates (**3a**–**m**) as colorless oils.
22. *Representative characterisation data for FLT phosphoramidates:* 3′-Deoxy-3′-fluorothymidine 5′-*O*-1-naphthyl-(neopentoxy-alaninyl)-phosphate (**3k**). This compound was synthesized according to the procedure above in 57% yield. The ratio of the diastereoisomers (d/s) at the phosphorous atom was 1/0.7 (^{1}H NMR analysis). ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.62 (br s, 1H, NH of one d/s), 8.58 (br s, 1H NH of one d/s), 8.05 (m, 1.7H, ArH), 7.86 (m, 1.7H, ArH), 7.68 (m, 1.7H, ArH), 7.56–7.49 (m, 5.1H, ArH), 7.42–7.35 (m, 2.7H, ArH+H5 of both d/s), 7.29 (dd, J = 6.0, 0.9 Hz, 0.7H, ArH), 6.29 (dd, J = 9.5, 5.2 Hz, 0.7H, H1′ of one d/s), 6.23 (dd, J = 9.4, 5.2 Hz, 1H, H1′ of one d/s), 5.17 (m, dd, J = 5.0, 3.5 Hz, 0.7H, H3′ of one d/s), 5.11 (m, dd, J = 5.0, 3.5 Hz, 1H, H3′ of one d/s), 4.50–4.45 (m, 0.7H, H4′ of one d/s), 4.43–4.30 (m, 4.4H, H4′ of one d/s, H5′ of both d/s), 4.19–4.10 (m, 1.7H, CH Ala of both d/s), 4.00–3.96 (dd, J = 9.0, 11.2 Hz, 1H, NH of one d/s), 3.88, 3.76 (AB system, J = 10.5 Hz, 2H, CH_2 neopentyl of one d/s), 3.84–3.80 (m, 0,7H, NH of one d/s), 3.86, 3.69 (AB system, J = 10.5 Hz, 1.4H, CH_2 neopentyl of one d/s), 2.46–2.35 (m, 2H, H2′a of both d/s), 1.82 (d, J = 0.9 Hz, 2.1H, CH_3 base of one d/s), 1.80 (d, J = 1.0 Hz, 3H, CH_3 base of one d/s), 1.70–1.62 (m, 1.7H, H2′b of both d/s), 1.42 (d, J = 7.0 Hz, 3H, CH_3 Ala of one d/s), 1.37 (d, J = 7.1 Hz,

2.1H, CH_3 Ala of one d/s), 0.93 (s, 9H, CH_3 neopentyl of one d/s), 0.90 (s, 6.3H, CH_3 neopentyl of one d/s). ^{13}C NMR (126 MHz, $CDCl_3$): δ 173.5 (d, J = 7.3 Hz, C═O of one d/s), 173.3 (d, J = 7.3 Hz, C═O of one d/s), 163.39 (Cq), 163.32 (Cq), 150.07 (Cq), 150.05 (Cq), 146.3 (d, J = 6.9 Hz, Cq), 146.1 (d, J = 6.8 Hz, Cq), 135.0 (C6 of one d/s), 134.8 (C6 of one d/s), 134.7 (Cq), 128.06, 128.04, 126.8, 126.56, 126.55 (CAr), 126.28, 126.23, 126.21, 126.16 (Cq), 125.46, 125.42, 125.40, 125.3, 125.2, 121.0, 120.9, 115.40, 115.3, 115.1, 115.08 (CAr), 111.44 (C5 of one d/s), 111.39 (C5 of one d/s), 93.4 (d, J = 179.3 Hz, C3′ of one d/s), 93.2 (d, J = 179.7 Hz, C3′ of one d/s), 85.0 (C1′ of one d/s), 84.8 (C1′ of one d/s), 82.9 (dd, J = 7.7, 27.0 Hz, C4′ of one d/s), 82.8 (dd, J = 7.7, 26.8 Hz, C4′ of one d/s), 74.98 (CH_2 neopentyl), 74.95 (CH_2 neopentyl), 66.1 (dd, J = 5.1, 11.3 Hz, C5′ of one d/s), 65.9 (dd, J = 5.2, 10.9 Hz, C5′ of one d/s), 50.58 (d, J = 1.5 Hz, CH Ala of one d/s), 50.50 (CH Ala of one d/s), 37.9 (d, J = 20.9 Hz, C2′ of one d/s), 37.8 (d, J = 20.9 Hz, C2′ of one d/s), 31.3 (Cq of one d/s), 31.4 (Cq of one d/s), 26.27 (CH_3 neopentyl of one d/s), 26.26 (CH_3 neopentyl of one d/s), 21.2 (d, J = 4.6 Hz, CH_3 Ala of one d/s), 21.1 (d, J = 5.2 Hz, CH_3 Ala of one d/s), 12.42 (CH_3 base of one d/s), 12.24 (CH_3 base of one d/s). ^{19}F NMR (471 MHz, $CDCl_3$): δ −174.92, −175.21. ^{31}P NMR (202 MHz, $CDCl_3$): δ 3.18, 3.03. MS (ES+) m/z: 614.20 (M+Na+). Reverse HPLC eluting with H_2O/MeOH from 90/10 to 0/100 over 25 min: t_R = 39.91 and 58.53 min (98%).

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25. *General method for synthesis of FLT phosphorodiamidates (**5a**–**b**).* Et_3N (1.05 equiv) was added dropwise to a solution of FLT (**1**) in anhydrous THF (0.2 M). The reaction mixture was stirred at rt for 30 min and was then cooled down to −78 °C before addition of $POCl_3$ (1.05 equiv) dropwise. The reaction mixture was stirred at −78 °C for 1 h before addition of the appropriate amino acid (5 equiv), DCM (4–5 mL) and Et_3N (10 equiv). The reaction mixture was stirred at rt for 16–27 h before evaporation of solvents to dryness. Purification of the crude by column chromatography ($CHCl_3$/MeOH 100/0 to 95/5) followed by a preparative TLC ($CHCl_3$/MeOH 95/5) gave the desired diamidate prodrug as a colorless oil.
26. *Representative characterisation data for FLT phosphorodiamidates:* FLT-5′-*O*-bis[(benzoxy-L-alaninyl)] phosphate (**5a**). This compound was synthesized according to the procedure above in 10% yield. ^{1}H NMR (500 MHz, MeOD): δ 7.54 (d, J = 1.2 Hz, 1H, H6), 7.37–7.30 (m, 10H, ArH), 6.26 (dd, J = 9.3, 5.5 Hz, 1H, H1′), 5.24 (dd, J = 5.0, 3.5 Hz, 1H, H3′), 5.14–5.11 (m, 4H, 2 × CH_2Ph), 4.33–4.26 (m, 1H, H4′), 4.14–4.06 (m, 2H, H5′), 3.98–3.92 (m, 2H, 2 × CH Ala), 2.50–2.41 (m, 1H, H2′-a), 2.30–2.17 (m, 1H, H2′-b), 1.87 (d, J = 1.2 Hz, 3H, CH_3 base), 1.38 (dd, J = 7.2, 0.6 Hz, 3H, CH_3 Ala), 1.34 (dd, J = 7.2, 0.8 Hz, 3H, CH_3 Ala). ^{13}C NMR (126 MHz, MeOD): δ 175.3 (d, J = 4.7 Hz, C═O), 175.2 (d, J = 6.0 Hz, C═O), 166.2 (C═O), 152.2 (C═O), 137.3 (C6), 137.2 (Cq), 129.61, 129.59, 129.38, 129.34 (CAr), 112.15 (C5), 95.1 (d, J = 176.6 Hz, C3′), 86.3 (C1′), 84.6 (dd, J = 8.3, 26.3 Hz, C4′), 67.98 (CH_2Ph), 67.96 (CH_2Ph), 66.0 (dd, J = 11.0, 5.2 Hz, C5′), 51.1 (CH Ala), 38.5 (d, J = 20.9 Hz, C2′), 20.7 (d, J = 6.2 Hz, CH_3 Ala), 20.6 (d, J = 6.5 Hz, CH_3 Ala), 12.6 (CH_3 base). ^{19}F NMR (471 MHz, $CDCl_3$): δ −176.58. ^{31}P NMR (202 MHz, $CDCl_3$): δ 13.69. MS (ES+) m/z: 669.23 (M+Na^+). Reverse HPLC eluting with H_2O/MeOH from 90/10 to 0/100 in 25 min: t_R 20.97 min (95%).
27. *Anti-HIV activity assays:* Inhibition of HIV-1(III_B)- and HIV-2(ROD)-induced cytopathicity in CEM cell cultures was measured in microtiter 96-well plates containing ~3 × 10^5 CEM cells/mL infected with 100 $CCID_{50}$ of HIV per milliliter and containing appropriate dilutions of the test compounds. After 4–5 days of incubation at 37 °C in a CO_2-controlled humidified atmosphere, CEM giant (syncytium) cell formation was examined microscopically. The EC_{50} (50% effective concentration) was defined as the compound concentration required to inhibit HIV-induced giant cell formation by 50%.
28. *Cytostatic activity assays:* All assays were performed in 96-well microtiter plates. To each well were added (5–7.5) × 10^4 tumour cells and a given amount of the test compound. The cells were allowed to proliferate for 48 h (murine lymphocytic CEM and human leukaemia L1210 cells) at 37 °C in a humidified CO_2-controlled atmosphere. At the end of the incubation period, the cells were counted in a Coulter counter. The IC_{50} (50% inhibitory concentration) was defined as the concentration of the compound that inhibited cell proliferation by 50%.