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Monitoring cellular accumulation of 3′-deoxy-3′-fluorothymidine (FLT) and its monophosphate metabolite (FLT-MP) by LC–MS/MS as a measure of cell proliferation *in vitro*

Wenlin Li[a], Marcela Araya[b], Mark Elliott[c], Xiaolin Kang[a], Phillip M. Gerk[b], Matthew S. Halquist[b], H. Thomas Karnes[b], Cathy Zhang[c], Peter J. O'Brien[a,*]

[a] Department of Pharmacokinetics, Dynamics, & Metabolism, Pfizer Worldwide Research and Development, San Diego, CA 92121, USA
[b] Department of Pharmaceutics, Virginia Commonwealth University, Richmond, VA 23298, USA
[c] Oncology Research Unit, Pfizer Worldwide Research and Development, San Diego, CA 92121, USA

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ABSTRACT

Accurate measurement of *in vitro* cell growth is critical for oncology drug development, but cell counting and the most accurate indirect proliferation assays are impractical. Here, we describe a robust alternative method that monitors proliferating cell thymidine kinase 1 (TK1) activity via LC–MS/MS quantification of 3′-deoxy-3′-fluorothymidine (FLT) and its monophosphate metabolite FLT-MP. LNCaP prostate cancer cells were cultured at four densities (20,000; 10,000; 5000; and 500 cells/well) and incubated with 2000 ng/mL FLT in multi-well plates. Internal standards were FLT-d3 for FLT and d4-thymidine for FLT-MP. In culture medium, peak area ratios of FLT to FLT-d3 and FLT-MP to d4-thymidine were linear over the range 0.25–100 ng/mL ($r^2 \geq 0.998$). Accuracy for quality controls was between −7.3% and 6.3% for FLT, and from −3.3% to 1.7% for FLT-MP. Quality control precision was from 2.4% to 5.7% for FLT and 3.2% to 7.5% for FLT-MP. The limit of quantification was 0.25 ng/mL, with good control results (precision of 9.6% for FLT and 14.8% for FLT-MP). FLT-MP formation was linearly proportional to cell number from 500 to 20,000 cells/well 1 h after FLT addition. FLT-MP and ATP generation were comparable in LNCaP cells exposed to cell cycle inhibitor drugs (Spearman $r = 0.925$, $p < 0.0001$), demonstrating assay suitability for drug screening. This fit for purpose method is amenable to analysis of tumor tissue extracts, and should enable direct assessment of *in vitro–in vivo* relationships in animal models of cancer.

1. Introduction

Understanding rates of cancer cell proliferation is critical for oncology drug development, but the field relies upon relatively few analytical methods for determining *in vitro* cell proliferation rates [1]. Cell counting and direct measurements of tumor mass are the "gold-standard" measures of cell proliferation, but are not practical in most pharmaceutical settings. The most common alternative, indirect quantification of DNA synthesis via the incorporation of [^{3}H]-thymidine or bromodeoxyuridine (BrdU) into DNA, can offer a high degree of sensitivity, but available methods are time consuming and labor-intensive, and are susceptible to experimental artifacts induced by long labeling incubations [2–7]. Cellular processes that are associated with cell proliferation and tend to increase proportionally with the number of proliferating cells in a sample can be measured using higher throughput methods. Such assays include DNA or nuclear staining; cellular reduction of tetrazolium salts, resazurin, or other indicator dyes as a measure of global metabolic processes; determination of cellular ATP concentrations by bioluminescence; and measurements of cellular oxygen consumption [1]. While these approaches can accurately reflect cell viability, energetics, and intermediary metabolism, most do not differentiate between quiescent and proliferating cells, and use conditions that can alter cell biology [5,8–10]. Though perhaps suitable for some early stage drug screens, poorly selective proliferation assays are less useful for later stage activities like lead optimization, especially in the development of narrowly targeted anticancer drugs where minimizing off-target pharmacology is a priority [11]. Furthermore, most *in vitro* proliferation assays cannot readily be translated for use in animal models and patient samples, where characterization of tumor growth rates can be critical for understanding drug responses. Histology, cytometry, and some of the methods described above are amenable to *ex vivo* analysis of tumor cell proliferation in excised tumor tissues, but these methods are limited by the availability of samples, and adequate cell numbers are often difficult to obtain. Thus, a rapid, sensitive

* Corresponding author. Tel.: +1 858 638 3734; fax: +1 858 526 4236.
E-mail address: Peter.O'Brien2@pfizer.com (P.J. O'Brien).

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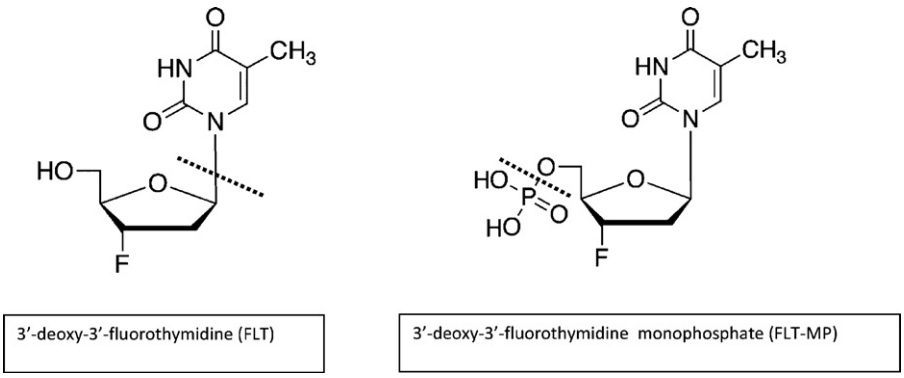

Fig. 1. Chemical structures of 3′-deoxy-3′-fluorothymidine (FLT) (Left) and 3′-deoxy-3′-fluorothymidine monophosphate (FLT-MP) (Right), with product ion fragmentation sites indicated by dashed lines.

assay that is translatable from cells to tissues is needed for unbiased monitoring anti-proliferative treatment responses in drug development.

The recent development of thymidine analog imaging agents like ^{18}F-3′-deoxy-3′-fluorothymidine ([^{18}F]-FLT) permits real-time, *in vivo* monitoring of tumors and other cells undergoing DNA synthesis via Positron Emission Tomography (PET imaging) [12]. Like the DNA labeling approaches described above, FLT PET permits the indirect monitoring of cellular thymidine kinase (TK1) activity. TK1 is expressed in normal cells during the G1-S phase of the cell cycle, and is critical to the salvage pathway of pyrimidine nucleotide biosynthesis during DNA replication [13]. TK1-mediated phosphorylation traps thymidine and its analogs in cells, where they accumulate over time. In tissues, TK1 activity is therefore reflective of the mass of proliferating cells, making it a useful marker in the diagnosis of certain types of cancer. For example, the activity of thymidine kinase is two orders of magnitude higher in A549 human lung carcinoma cells compared to normal lung tissue [14]. Phosphorylated FLT (FLT-MP) is not incorporated into DNA, but its monophosphate metabolite ([^{18}F]-FLT-MP) rapidly accumulates in the cell, a process that enables its detection in [^{18}F]-FLT PET imaging [14,15]. Anti-proliferative drugs can reduce the accumulation of [^{18}F] (as FLT and FLT-MP) in cells in a dose-dependent manner, demonstrating that the degree of FLT uptake and FLT-MP formation in specific tissues can reflect cellular proliferation and be used as a biomarker in animal models of cancer [16]. Although [^{18}F] FLT-MP accumulation is potentially useful as a translatable biomarker, the radioactive half-life of ^{18}F (1.82 h) requires that [^{18}F]-FLT be synthesized and administered quickly to animals and patients, and requires dynamic recalibration of the remaining radioactivity for accurate image interpretation [17,18]. Furthermore, current imaging methods cannot distinguish between parent tracer and FLT-MP metabolites, information that would further clarify the proliferative status of tumors.

The ability to rapidly and safely quantitate FLT and its metabolites *in vitro* can potentially bridge laboratory and clinical studies by providing quantitative, translatable measurement of cell proliferation. This approach is feasible using modern LC–MS/MS methods, which allow extremely specific and sensitive, non-radioactive quantitation of small molecule drugs and metabolites, including nucleoside analogs like FLT. The potential for developing a simple extraction and analysis procedure for measuring FLT and its metabolites by LC–MS/MS provides a feasible alternative for higher throughput *in vitro* TK1 activity assays, and offers a mechanistically relevant bridge between laboratory and *in vivo* studies. Our aim in the studies described here was to develop and validate a suitable non-isotopic alternative assay for measuring TK1 activity in cells via quantitation of FLT and FLT-MP in cell extracts.

2. Materials and methods

2.1. Chemicals and reagents

Acetonitrile and methanol HPLC Grade were purchased from Honeywell Burdick & Jackson (Muskegon, MI, USA). Thymidine was obtained from Sigma–Aldrich (St. Louis, MO, USA). 3′-Deoxy-3′-fluorothymidine (Fig. 1), 3′-deoxy-3′-fluorothymidine-5′-monophosphate disodium salt (FLT-MP), and 3′-deoxy-3′-fluorothymidine-d3 were purchased from Toronto Research Chemicals Inc. (TRC, Toronto, ON, Canada). D4-thymidine was obtained from CDN isotopes, Inc. (Thaxted, United Kingdom). Formic acid was obtained from Sigma–Aldrich (St. Louis, MO, USA). High purity water was obtained in-house using a NANOpure DIamond Life Science ultrapure water System from Barnstead International (Dubuque, IA, USA). CellTiter-Glo® luminescent cell viability assay kit was obtained from Promega (Madison, WI, USA). CDK4 Inhibitor III (5-(N-(4-methylphenyl) amino)-2-methyl-4,7-dioxobenzothiazole) and Aurora Kinase Inhibitor III (cyclopropanecarboxylic acid-(3-(4-(3-trifluoromethyl -phenylamino)-pyrimidin-2-ylamino)-phenyl)-amide) were obtained from EMD Biosciences, Inc. (La Jolla, CA) and prepared as stock solutions by dissolving in cell culture grade dimethyl sulfoxide (DMSO), which was obtained from Sigma–Aldrich.

2.2. Cell culture

LNCaP prostate carcinoma cells (CRL-1740) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA), and grown in complete growth medium consisting of RPMI-1640 Medium from ATCC supplemented with 10% heat treated fetal bovine serum (FBS, Sigma) without antibiotics. Cells were incubated at 37 °C in an atmosphere of 95% air and 5% CO_2. Cells (passage 10) were seeded into a Corning 3997 96-well plate at 4 densities (20,000; 10,000; 5,000; and 500 cells/well). Cell counts were confirmed by counting in a hemacytometer using trypan blue exclusion to assess cell viability. Cell attachment and low seeding density were confirmed by inverted light microscopy, and cells were allowed to attach overnight (18 h) in 100 μL medium. For determining FLT uptake and FLT-MP formation, 100 μL complete growth medium containing 4000 ng/mL FLT was added to achieve final concentration of 2000 ng/mL in each well (200 μL total volume). The cells were incubated at 37 °C for 5 min, 1, 2 and 4 h; then the medium was removed to a collection plate without disturbing attached cells. 250 μL of methanol was added to the cells in each well. Both plates were sealed with adhesive foil and stored at −20 °C until analysis. LC/MS/MS determinations were made in

quadruplicates for the 4 levels of cell density and 4 incubation times.

LNCaP cell FLT-MP formation was compared with an alternative endpoint assay, cellular ATP generation as follows: 96 well tissue culture plates were coated with poly-D-lysine and dried prior to seeding of 10,000 LNCaP cells per well. Cells were grown overnight in 100 μL complete growth medium. The next morning, medium was aspirated and replaced with 100 μL RPMI 1640 medium without supplements (*i.e.*, starvation medium), and incubated 4 h as above [19]. DMSO (vehicle) or drug solution (0.01, 0.1, 1.0 and 10 μM final concentration) was added to each well, so that the final concentration of DMSO did not exceed 0.1%. Thus treated, cells were incubated for 1 h, re-fed (stimulated) by adding one volume of complete growth medium containing 10% fetal bovine serum (final serum concentration 5%), then cultured for up to five days. Basal (unstimulated) cell growth was monitored using cells re-fed with starvation medium containing 0.1% DMSO, allowing comparisons of cells cultured for the same period of time in the presence and absence of serum and drug. LNCaP cell FLT-MP generation was compared with ATP generation, the latter measured using the CellTiter-Glo® cell viability assay per manufacturer instructions. A cell stimulation index was calculated for FLT-MP generation or ATP generation by dividing the assay signal from stimulated cells by that of unstimulated (starved) cells.

Statistical analysis of results was performed using Graphpad Prism (Graphpad Software, LA Jolla, CA, USA).

2.3. Chromatographic conditions

The HPLC system consisted of a Shimadzu (Kyoto, Japan) controller SCL-10A VP, pumps LC-10AD VP, solvent degasser DGU14A, auto-sampler HTS PAL from CTC Analytics (Zwingen, Switzerland) and a Eppendorf CH-30 column heater (Westbury, NY, USA). Chromatographic separation was achieved on an Aquasil C18 column (100 mm × 2.1 mm, 5 μm) (Thermo Scientific, Waltham, MA, USA), maintained at 45 °C. A Phenomenex C18 guard column (4.0 mm × 2.0 mm) was used to extend life of the analytical column. The mobile phases consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The total running time was 7 min with a gradient elution at a flow rate of 0.4 mL/min. Gradient conditions include: 0–35% mobile phase B over 3.5 min, then 80% B to 4.3 min., hold at 80% until 5.0 min, and finally equilibrate for 1.5 min at the original condition of 100% A. The injection volume was 20 μL.

Table 1
The optimum HPLC–MS/MS conditions (CE: collision energy and DP: declustering potential) for analytes and internal standards.

Compound	Q1 mass (Da)	Q3 mass (Da)	DP	CE
FLT	245.1	127.1	30	16
FLT-d3	248	130.2	30	16
FLT-MP	325.3	81.2	40	22
D4-thymidine	247.3	131.3	30	16

2.4. Mass spectrometry conditions

Mass spectra were obtained using an Applied Biosystems SCIEX API 4000 QTrap Mass Spectrometer operated in the positive electrospray ionization (ESI) mode. Tuning and optimization of mass spectrometer parameters were performed for the analytes and internal standard (IS, see below) by direct infusion of a 1 μg/mL standard solution at a flow rate of 20 μg/mL. The multiple reaction monitoring (MRM) transitions, declustering potential (DP) and collision energy (CE) for all the compounds are listed in Table 1. The remaining parameters were as follows: ion source voltage (IS) 5500, curtain gas (CUR) 25, gas 1 (GS1) 40, gas 2 (GS2) 40, entrance potential (EP) 10, collision exit potential (CXP) 10, and the ion source temperature was set at 480 °C. Nitrogen was used as curtain gas. Nitrogen was obtained from a Parker Balston Tri Gas Generator LCMS-5000 (Haverhill, MA, USA). Data were acquired with Analyst software, Version 1.5.

2.5. Preparation of stock solutions, standards and quality control samples

Standard matrix was prepared by adding 1 mL blank culture media to 9 mL methanol stock solutions were prepared by dissolving FLT and FLT-MP in methanol to yield 500 μg/mL. Working solutions were prepared by further diluting stock solution in methanol to obtain the following concentrations: 5, 10, 20, 50, 100, 200, 500, 1600 and 2000 ng/mL. Quality control (QC) standards of 5, 15, 80 and 1600 ng/mL were similarly prepared. The working solutions and QC samples were kept at −20 °C until analyzed. IS stock solution was prepared by dissolving FLT-d3 and D4-thymidine in methanol to a final concentration 200 ng/mL. Stock solutions, QC solution and IS solutions were stored at −20 °C.

Table 2
Inter-assay and intra-assay precision and accuracy for FLT and FLT-MP.

	Nominal concentration (ng/mL)			
	0.25	0.75	4.00	80.00
Observed FLT concentration (ng/mL)				
Inter-assay mean ± SD	0.247 ± 0.0238	0.769 ± 0.0331	4.12 ± 0.160	78.88 ± 2.36
Inter-assay precision (%RSD)	9.6	4.3	3.9	3.0
Inter-assay accuracy (%DFN)	1.1	2.5	3.0	1.4
Intra-assay mean ± SD (n = 6)	0.240 ± 0.0130	0.772 ± 0.0308	4.15 ± 0.0741	78.02 ± 2.87
Intra-assay precision (%RSD)	5.4	4.0	1.8	3.7
Intra-assay accuracy (%DFN)	−4.1	2.9	3.8	−2.5
Observed FLT-MP concentration (ng/mL)				
Inter-assay mean ± SD	0.271 ± 0.0401	0.746 ± 0.0448	4.00 ± 0.121	82.18 ± 4.22
Inter-assay precision (%RSD)	14.8	6.0	3.0	5.1
Inter-assay accuracy (%DFN)	8.3	0.6	0.0	2.7
Intra-assay mean ± SD (n = 6)	0.246 ± 0.0200	0.753 ± 0.0403	4.00 ± 0.0812	80.37 ± 5.04
Intra-assay precision (%RSD)	8.2	5.3	2.0	6.3
Intra-assay accuracy (%DFN)	−1.7	0.4	0.1	0.5

SD: standard deviation; %DFN: percent deviation from nominal value; %RSD: percent relative standard deviation.

2.6. Sample processing

Calibration standards were prepared by adding 10 μL of the corresponding working solution or QC to 190 μL standard matrix to a 1.5 mL microcentrifuge tube. A 200 μL aliquot of methanol-extracted cells was transferred to a 1.5 mL microcentrifuge tube, and 50 μL of internal standard mixture was added. Samples were vortexed for 2 min and centrifuged for 5 min at 4 °C at 10,621 × *g* (10,000 rpm). Supernatant (240 μL) was transferred into a 10 mL glass conical tube and evaporated under nitrogen stream (50 °C for 15 min at 25–30 psi). Samples were reconstituted in 50 μL of HPLC grade water containing 1.0 mM Citric Acid and 0.5 mM EDTA as a chelator to inactivate phosphatases after cell lysis [20]. Samples were transferred to 96 well plates, from which 20 μL sample was injected to the LC–MS/MS system.

2.7. Matrix effects

In order to evaluate matrix effects, a post-column infusion study was conducted. A 100 ng/mL solution FLT/FLT-MP in methanol was prepared and continuously infused at 20 μL/min post-HPLC column directly into the mass spectrometer using a "tee" connection. After stabilization of baseline a blank sample was injected. A blank sample is an extracted standard matrix (prepared by adding 1 mL blank culture media to 9 mL methanol) as described in Section 2.6.

2.8. Validation procedures

Validation of the assay was performed according to the FDA guidelines for Bioanalytical Method Validation [21]. Validations runs containing duplicate calibration standards, blank samples, blank samples spiked with internal standard and replicates of QC samples were run on three separate days.

2.8.1. Linearity and LLOQ

Nine calibration standards with concentrations of 5, 10, 20, 50, 100, 200, 500, 1600 and 2000 ng/mL were extracted in duplicate and analyzed in 3 independent runs. Calibration curves were fitted using the linear regression of the ratio of the peak area response of the analyte and the internal standard versus concentration. For each calibration curve, the back calculated concentrations were required to be within ±15% of nominal concentration (DFN) except at the limit of quantification (LLOQ) where it could be within ±20%. The lower limit of quantification (LLOQ) was the concentration of FLT/FLT-MP at which the analyte response signal was at least a five times that of the blank.

2.8.2. Accuracy and precision

Accuracy and precision were determined from QC samples for three independent runs. A criterion of ±15% of the nominal concentration was used to assess accuracy (±20% for LLOQ) and precision was expressed as %RSD, which should not exceed ±15% (±20% for LLOQ). Intra-assay precision and accuracy were determined from 6 replicates of each QC sample on a single assay. Inter-assay precision and accuracy were determined by analyzing three different validation runs.

2.8.3. Recovery and carryover

Recovery during the extraction procedure was determined by comparing peak areas. Samples were spiked with FLT/FLT-MP at three levels LQC, MQC and HQC (0.75, 4.0 and 80.0 ng/mL). These samples were extracted and compared to blank samples spiked post-extraction with the same final concentrations of FLT/FLT-MP. Carryover was evaluated by injecting LQCs after HQCs ($n = 6$) and also by injecting a reconstitution solution following the highest calibration standard.

3. Results and discussion

3.1. LC–MS/MS

LC–MS/MS conditions were optimized for all analytes of interest, allowing their quantification with a total run time of 7 min. Retention times were 3.66, 3.25, 3.65 and 3.25 min for selected transitions of FLT (m/z 245.1 → 127.1), FLT-MP (m/z 325.3 → 81.2), FLT-d3 (m/z 248 → 130.2) and D4-thymidine (m/z 247.3 → 131.3), respectively. Positive electrospray ionization allowed for the formation of protonated forms of each analyte, which occurred on the secondary amine of FLT and FLT-MP, and their respective internal standards. Selection of product ions was based upon intensity and reproducibility. FLT and FLT-MP fragmentation patterns are shown in the structures (Fig. 1) designated by a dashed line. Representative chromatograms of an extracted and blank sample enriched with FLT and FLT-MP at 0.25 ng/mL are shown in Fig. 2.

3.2. Validation of the analytical method

3.2.1. Linearity

The peak area ratio of FLT to FLT-d3 and FLT-MP to D4-thymidine in culture medium was linear over the range 0.25–100 ng/mL. The calibration curves for both FLT and FLT-MP yielded a mean correlation coefficient ≥0.998. A weighting factor of $1/x^2$ and $1/x$ was used for FLT and FLT-MP, respectively. Precision of the calibration standards, measured as the percent relative standard deviation for the mean back-calculated values, ranged from 2.4% to 5.7% for FLT and 3.2% to 7.5% for FLT-MP.

3.2.2. Accuracy and precision

The percent deviation from nominal (%DFN) for the mean back-calculated values of the calibration standards were between −7.3% and 6.3% for FLT, while %DFN ranged from −3.3% to 1.7% for FLT-MP. The limit of quantification (LLOQ) was established at 0.25 ng/mL for both FLT and FLT-MP with a precision of 9.6% for FLT and 14.8% for FLT-MP. The LLOQ was reproducible with an accuracy of −1.1% for FLT and 8.3% for FLT-MP. Inter-assay and intra-assay precision and accuracy for FLT and FLT-MP quality control samples are shown in Table 2.

3.2.3. Extraction recovery

The mean extraction recovery for FLT in culture media determined at LQC, MQC and HQC levels were 57.6%, 56.8% and 67.8% respectively. The results for FLT-MP show a mean extraction recovery of 49.9%, 51.0% and 58.1% for LQC, MQC and HQC respectively. The post-extraction addition study revealed that this relatively low recovery was likely due to relatively low extraction recovery from the sample preparation process. Chromatographic conditions were optimized based on ion suppression profiles, and the matrix was duplicated in unextracted samples. Therefore it is not likely that low recovery was due to a matrix effect.

3.2.4. Matrix effects

Matrix effects were evaluated with post-column infusion of a 100 ng/mL FLT/FLT-MP solution. Fig. 3A shows the ion profiles of the infused solution upon injection of a processed blank sample. Chromatographic conditions were modified to shift the retention time of the analytes out of the ion suppression (Fig. 3B). The post-extraction addition study is discussed under Section 3.2.3, which reveals the recovery and thus relative amounts of ion suppression present.

3.2.5. Carryover and autosampler stability

All LQCs injected after the HQCs were reproducible with an accuracy of 2.9% for FLT and 0.4% for FLT-MP, and precision of 4.0% and

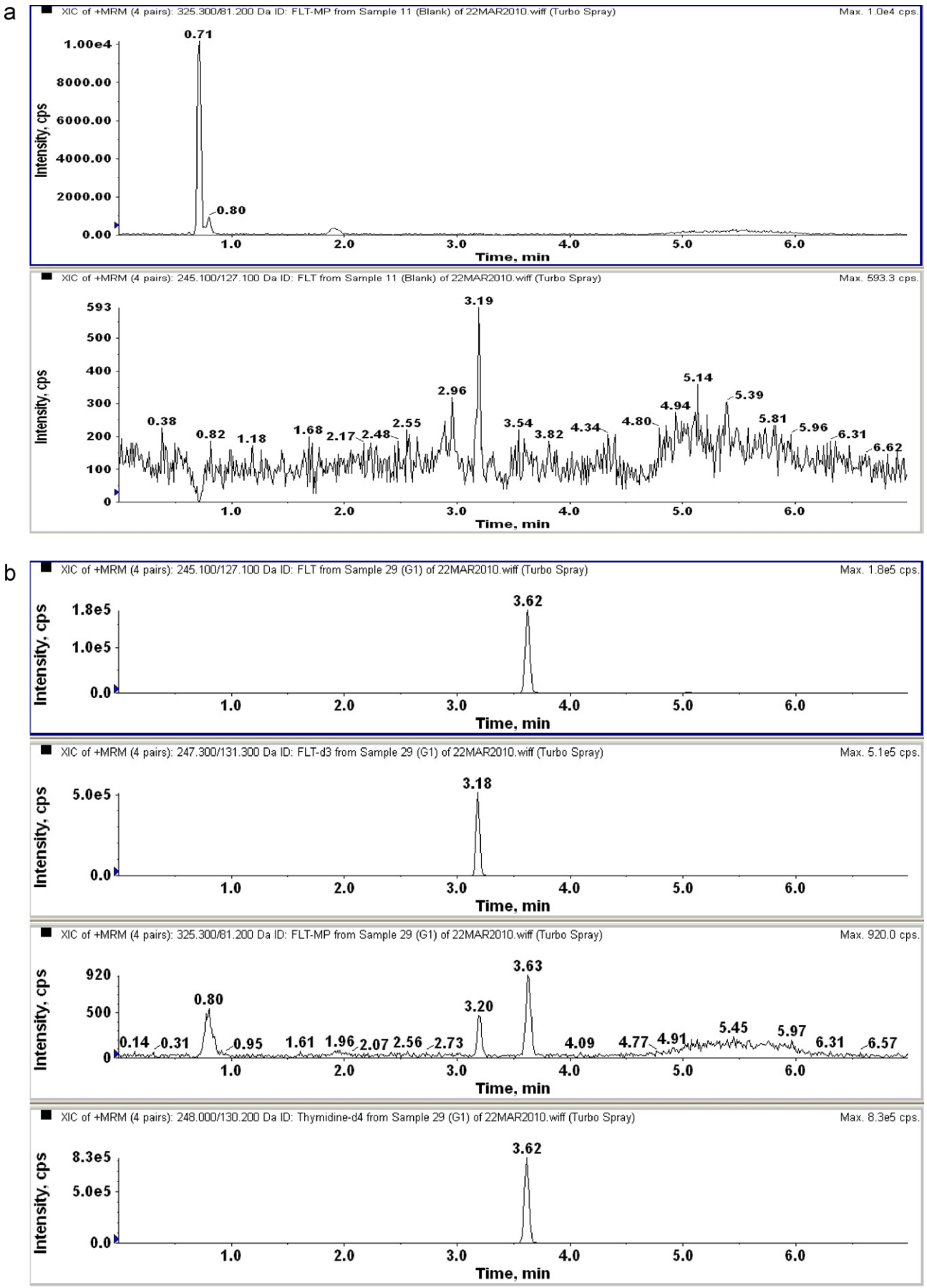

Fig. 2. (A) Typical blank chromatogram of FLT and FLT-MP. (B) Typical sample chromatogram of FLT, FLT-d3, FLT-MP and D4-thymidine of 20,000 cells plated.

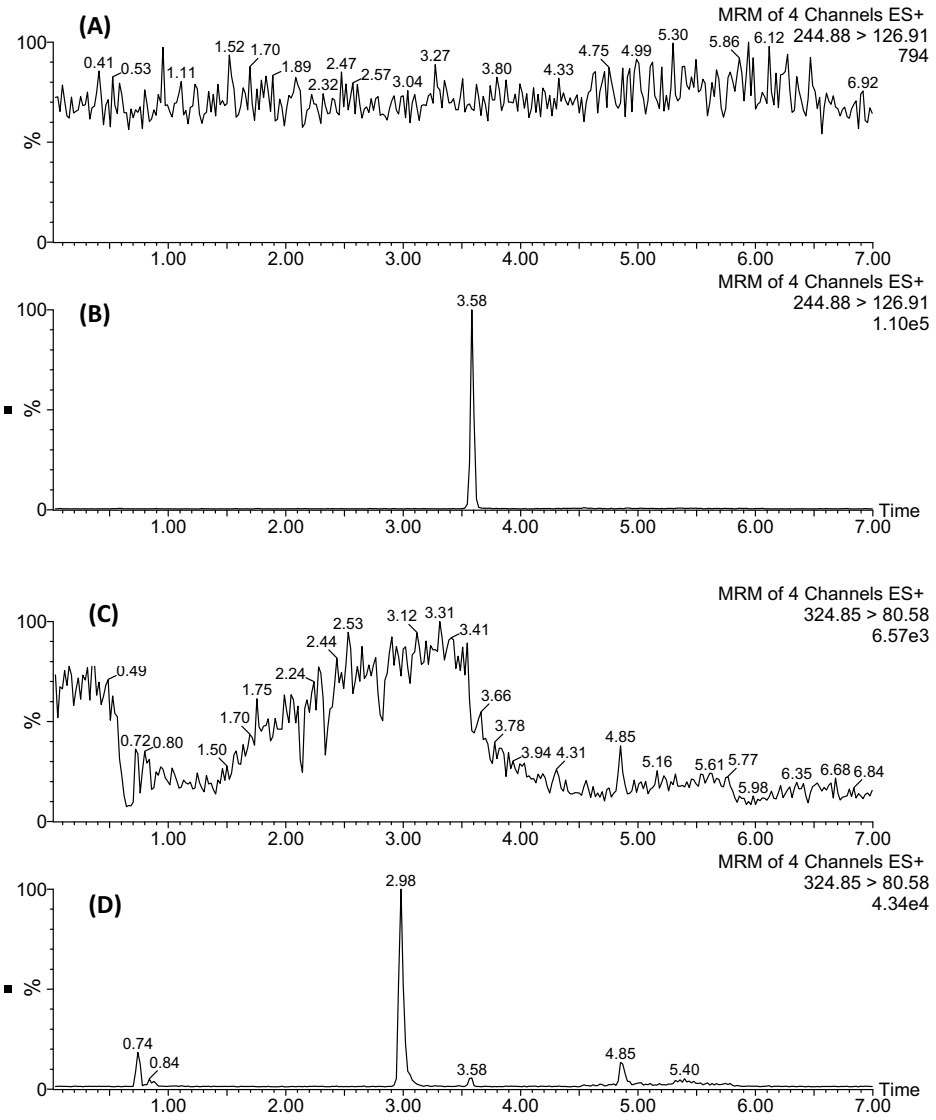

Fig. 3. Results of post-column infusion. (A) Ion profile upon injecting a processed blank sample monitoring FLT transition (244.88 > 126.91). (B) Representative chromatogram showing retention time for FLT. (C) Ion profile upon injecting a processed blank sample monitoring FLT-MP transition (324.85 > 80.58). (D) Representative chromatogram showing retention time for FLT-MP.

5.3% for FLT and FLT-MP, respectively. The reconstitution blank injected after the highest calibration standard was found to be below detection limits for FLT and FLT-MP. Autosampler stability was evaluated for storage of quality controls for 50 h at 5 °C and determined to have acceptable precision and accuracy (±15%) for all quality controls.

3.3. Incurred sample analysis

The validated method was applied to quantify FLT-MP in LNCaP prostate cancer cells after FLT treatment. Analysis of cell lysates demonstrated rapid FLT-MP formation, which was quantifiable at a cell density as low as 500 cells/well (Fig. 4A) following 1 h of incubation with FLT (2000 ng/mL). FLT-MP levels correlated well ($Y = (0.000611 \pm 0.000010) \times X$; $r^2 = 0.992$) with the number of cells incubated with FLT for 1 h (Fig. 4B). Cell supernatants showed no detectable FLT-MP under the conditions tested, suggesting that cell lysis and leakage of FLT-MP into the culture supernatant was minimal (data not shown).

3.4. Comparison with established methods

In order to test the utility of the FLT-MP assay in anti-cancer drug screening, the method was compared with results obtained using the widely used CellTiter-Glo® cell viability assay. These studies were performed in LNCaP cells, since prostate cancer presents several critical challenges for *in vitro* drug screening. Firstly, in prostate cancer, the proliferative rate can be less than 10%, and prostate cancer cell lines like LNCaP are characteristically quiescent [22]. Although starvation–refeeding methods like those used here can increase the proliferative fraction of cultured prostate cancer cells, it can take up to five days for ATP generation assays to reproducibly discriminate untreated cells from those dying due to drug exposure (our unpublished observations). Furthermore, LNCaP cells adhere relatively loosely to tissue culture plastic compared to other cell lines, making them a challenging test case for the use of FLT-MP generation in screens of anti-proliferative drugs [23].

LNCaP cells were stimulated to proliferate in the presence of Aurora Kinase Inhibitor III, or CDK4 Inhibitor III, anti-proliferative drugs with well-characterized effects on prostate cancer cell lines [24–26]. In identical parallel assays, FLT-MP formation was

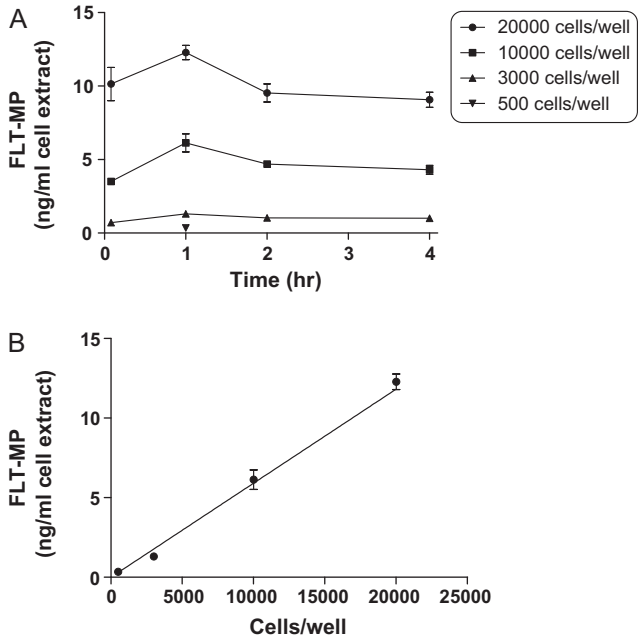

Fig. 4. Application of the method to determine LNCaP cell proliferation. (A) FLT-MP levels in LNCaP prostate cancer cell line after incubation with 2000 ng/mL FLT. Data are represented as mean ± SD of quadruplicate determinations. At 500 cells/well, FLT-MP was above the LLOQ (0.25 ng/mL) only at 1 h. (B) Correlation of FLT-MP levels in LNCaP prostate cancer cell line with number of incubated cells after 1 h incubation with FLT (2000 ng/mL). Data are represented as mean ± SD of quadruplicate determinations.

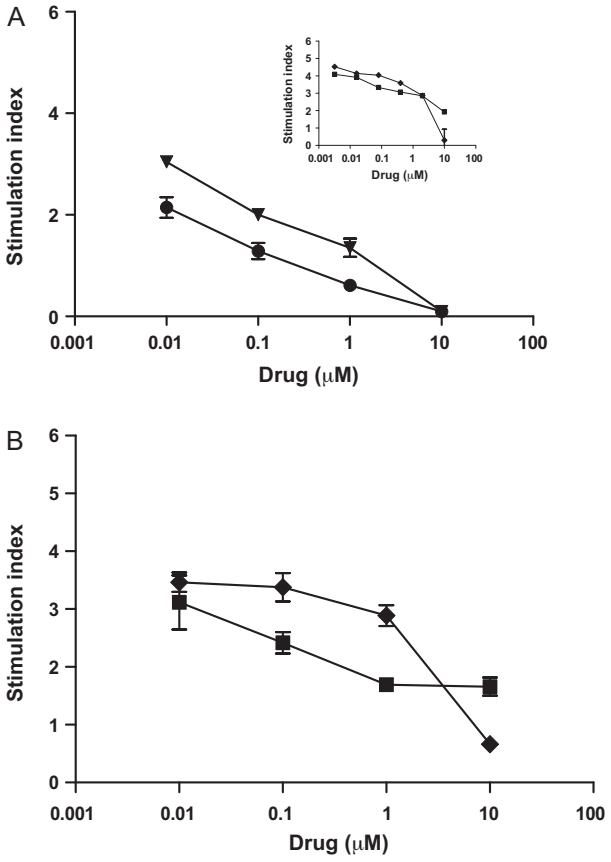

Fig. 5. Comparison of the method with ATP generation as a measure of cell viability. FLT-MP concentrations (A) or ATP generation (B) determined in LNCaP cells after incubation with CDK4 inhibitor III (squares) or Aurora kinase inhibitor III (diamonds). The stimulation index is calculated by dividing either FLT-MP or ATP concentration from re-fed (stimulated) cells with that from unstimulated (*i.e.*, starved), vehicle treated cells. Data are represented as mean ± SD of triplicate determinations. 10,000 LNCaP cells per well were serum starved then re-fed in the presence of drug or vehicle, incubated for up to 120 h, then assayed for FLT-MP or ATP generation as described in Section 2. Drug effects on FLT-MP accumulation were detectable at both 48 h (inset) and 120 h post-stimulation.

compared with cellular ATP generation, measured using the CellTiter-Glo® assay. ATP generation is well-correlated with the number of viable cells in a culture cell, and this endpoint assay is widely used as a surrogate measure of cell proliferation [27]. As shown in Fig. 5, results from the FLT-MP generation and the CellTiter-Glo® assays showed rank order correlation (Spearman $r = 0.925$, two tailed $p < 0.0001$). Although results from the two assays are consistent, these endpoint assays reflect fundamentally different cellular processes (*i.e.*, DNA replicative potential versus metabolic viability, respectively), and are unlikely to be truly interchangeable. This conclusion is supported by several reports comparing cell viability assays with pulse labeling of DNA [28–31]. Given these confounding factors, understanding the direct comparability of cell viability measurements and TK1 activity will require additional research. The data presented here nevertheless show that the two approaches are at least consistent and are potentially useful as complementary measurements in early drug development.

The method described here can be used to monitor even low numbers of cells, as in a microplate format compatible with high-throughput screening. This analytical approach may thus be useful for measuring TK1 activity in very small samples of human tissue, including biofluids and cell obtained from needle biopsies. The intended use of this method, measurement of tumor cell responses to anti-proliferative treatments, presents unusual analytical challenges that were addressed in this fit-for-purpose validation. Specifically, since successful anti-proliferative treatment would elicit a dose dependent effect on cell number, it is highly likely that each sample would have a distinct matrix composition. Even in cases where cell numbers are identical, the possibility remains that a given treatment would evoke differential effects on intermediary metabolism, again yielding a potentially infinite range of different matrices. To address this issue, we used a substitute matrix for the preparation of calibration standards. Although this method measures the xenobiotic metabolite FLT-MP, this compound can be considered a biomarker of cell or tissue functional response to treatment. Considered in that light, the decision to use a substitute matrix and accept consistent, if not quantitative recovery is consistent with accepted cell and tissue biomarker assay validation practices [32]. Our findings demonstrate that the production of FLT-MP is linearly proportional to cell number, strongly suggesting that matrix effects can be disregarded for this particular application. More advanced applications will require that this issue be revisited.

In its current configuration, this cellular TK1 activity assay requires that live cells be treated with FLT and immediately extracted in MeOH. Although this quenches the kinase reaction, FLT-MP degrades over time and must be analyzed within 50 h using a refrigerated autosampler to preserve sample integrity. The method validation described here demonstrates that the assay is suitable for the purpose of *in vitro* drug screening, but does not assure that the method is valid for more advanced applications. For example, the analysis of cell proliferation in tumor biopsies or explanted cells will likely require the use of additional internal standards, further examination of sample stability and matrix effects, and other potentially confounding biological factors.

Further studies are currently in progress to assess TK1 phosphorylation of FLT in additional tumor cell lines with the goal of establishing correlations between *in vitro* TK1 activity and tumor

growth *in vivo*, and to compare FLT-MP generation with other proliferation/viability assays.

4. Conclusions

A sensitive, accurate and precise procedure has been developed and fit-for-purpose validated for drug screening via the determination of 3′-deoxy-3′-fluorothymidine and 3′-deoxy-3′-fluorothymidine monophosphate in cell extracts by LC–MS/MS. This method was designed and validated to be fit for the purpose of early stage *in vitro* screening, and is intended to either replace or be complementary to existing, less selective cell viability assays. Good linearity was obtained with concentrations from 0.25 to 100 ng/mL. The method described here is likely to be selective for proliferating cells, since intracellular TK1 activity occurs in proliferating, but not resting or dying cells. Furthermore, cell cycle progression occurs on a time scale that is much longer than the FLT incubation time described here. This contrasts with DNA incorporation assays and some cell viability methods, which require overnight incubations that likely span more than one cell generation. The TK1 activity assay described here thus likely limits the impact of potentially serious confounding factors [5,8,9].

Finally, the ease of application of this method seems likely to further our understanding of the role of TK1 in cell proliferation. During assay optimization, for example, we noted that FLT-MP formation peaked 1 h after FLT addition. This metabolite accumulation was lower at later time points. In the absence of evidence for leakage of FLT-MP from cells during the times we examined, we attribute this observation to further metabolism of FLT-MP to its di- and tri-phosphate forms, neither of which would be detected in our method. It remains possible that other cellular processes drive these changes in intracellular FLT-MP, and investigations are underway to clarify this finding. The method described here offers a novel, non-radioactive approach for studying nucleoside metabolism, a major target of cancer chemotherapy.

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