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Imaging Pharmacodynamics of the alpha Folate Targeted Thymidylate Synthase Inhibitor BGC 945

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Abstract

The assessment of tissue-specific pharmacodynamics is desirable in the development of tumour-targeted therapies. Plasma deoxyuridine (dUrd) levels - a measure of systemic thymidylate synthase (TS) inhibition - has limited application for studying the pharmacodynamics of novel TS inhibitors targeted to the high affinity alpha-folate receptor (FR). Here we have evaluated the utility of [¹⁸F]fluorothymidine positron emission tomography ([¹⁸F]FLT-PET) for imaging the tissue pharmacodynamics of BGC 945, a FR-targeted antifolate TS inhibitor; the non-targeted antifolate BGC 9331 was used for comparison. TS inhibition by both drugs induced a concentration-dependent increase in [³H]thymidine uptake in FR-positive human epidermoid KB cells. Membrane associated equilibrative nucleoside transporter type 1 levels increased from 55720±6101 to 118700±5193 and 130800±10800 per cell at 100 µg/ml of BGC 9331 and BGC 945, respectively, suggesting this as a potential mechanism of increased nucleoside uptake. In keeping with these in vitro findings, tumour [¹⁸F]FLT accumulation in KB xenografts increased by 2-fold after drug treatment with maximal levels at 1-4 and 4-24 h following BGC 9331 and BGC 945 treatment, respectively. Of interest to FR-targeting, BGC 9331 but not BGC 945 induced accumulation of [¹⁸F]FLT uptake in intestine, a proliferative and TS-responsive tissue. For both drugs, quantitative changes in tumour [¹⁸F]FLT uptake were associated with increased tumour dUrd levels. In conclusion, we have validated the utility of [¹⁸F]FLT-PET to image TS inhibition induced by antifolates and demonstrated the tumour-specific activity of BGC 945. This imaging biomarker readout will be useful in the early clinical development of BGC 945.

Keywords

Antifolate; thymidylate synthase; pharmacodynamics; positron emission tomography; [¹⁸F]fluorothymidine

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INTRODUCTION

New agents with unique tumour targeting properties are being developed in a number of institutions. One such agent, the antifolate BGC 945, selectively binds to the α -folate receptor (α -FR), a cell-surface glycoprotein, with very high affinity, similar to that of folic acid ($K_d \sim 0.1 \text{ nM}$), a natural ligand. It is then transported into cells by receptor-mediated endocytosis where it acts as a potent inhibitor of thymidylate synthase (TS) ($K_i \text{ TS} = 1.4 \text{ nM}$), leading to cell death (1, 2). The α -FR is over-expressed in several cancers, particularly epithelial tumours such as ovarian carcinoma (up to 90% of cases). However, it has restricted expression in TS-responsive normal proliferating tissues such as gut and bone marrow, and this expression is limited to the apical surface membrane and therefore not assessable to FR-binding agents in the circulation (3, 4). Compared to other antifolates such as raltitrexed and plevitrexed (BGC 9331) which are taken up into cells predominantly via the ubiquitous reduced folate carrier (RFC) (5), BGC 945 has the potential to have a particularly high therapeutic index because of reduced activity on normal TS-responsive tissues. However, this efficacy is likely to be seen only in patients with tumours expressing the α -FR.

Pharmacodynamic biomarkers that report on modulation of a biological target and help define a biologically effective dose (BED) are increasingly being incorporated into early clinical development of targeted anticancer agents. With respect to TS inhibition, the most widely accepted biomarker is increased plasma deoxyuridine (dUrd) levels measured by high performance liquid chromatography (HPLC) or liquid chromatography mass spectrometry (LCMS) (6, 7). Elevated plasma dUrd, however, mainly reflects systemic TS inhibition in normal proliferating tissues rather than on tumour alone. This assay has been adapted to measure tumour dUrd levels and will be appropriate for BGC 945, but this will involve multiple core biopsies in clinical trials and hence be inconvenient for patient studies. Recently, our group reported a novel method for imaging TS inhibition by non-invasive positron emission tomography (PET) (8). A unique biochemistry of tissues in which TS inhibition leads to redistribution of the nucleoside transporter ENT1 to the cell membrane, provided the basis for using [^{18}F]fluorothymidine([^{18}F]FLT)-PET to image 5-fluorouracil-induced TS inhibition (8). In this manuscript, we have validated this imaging method for antifolates with BGC 9331 in time and dose and used the method for the first time to quantitatively evaluate tissue-specific TS inhibition by the antifolate, BGC 945.

MATERIALS AND METHODS

Antifolates and radiopharmaceutical

BGC 945 ((2) as the 6-*S*-trifluoroacetic acid salt) and BGC 9331 were supplied by BTG International, London UK. BGC 9331 was a 10mM solution, stored at 4°C and used within recommended storage dates for each batch. BGC 945 was provided in solid form and dissolved in 0.15 M NaHCO_3 for in vitro use and stored at -20°C as a 10 mM solution for up to 3 months. For in vivo use, BGC 945 was dissolved in 0.05 M NaHCO_3 and the pH of the dose solution adjusted to ~ 9 by addition of 1 M NaOH. [^{18}F]FLT was synthesised by radiofluorination of the 3-*N*-Boc-5'-*O*-dimethoxytrityl-3'-*O*-nosyl-thymidine precursor using a GE TracerLab MX synthesis module (GE Healthcare UK Limited, Little Chalfont, Buckinghamshire, UK) based on a method previously described by Oh et al. (9). All samples had > 98% radiochemical purity as determined by high performance liquid chromatography with radiochemical detection (radio-HPLC), and the specific radioactivity was 120–150 GBq/ μmol at the end of synthesis. [Methyl- ^3H]Thymidine (^3H Thd; 925 GBq/mmol, 37 MBq/ml) and [5- ^3H]-deoxyuridine ([5- ^3H]-dUrd; 925 GBq/mmol, 37 MBq/ml) were purchased from GE Healthcare UK Limited (Little Chalfont, Buckinghamshire, UK). [^3H]Nitrobenzyl mercaptopurine ribonucleoside (^3H NBMPR; also known as [^3H]-S-(p-

nitrobenzyl)-6-thioinosine; 555 GBq/mmol, 37 MBq.ml⁻¹) was purchased from Moravек Biochemicals Inc. (Brea, CA, USA).

Intrinsic uptake of nucleosides by tumour cells

[³H]Thd was used to assess TS-induced nucleoside uptake in cultured cells. For this, α-FR-positive human epidermoid KB cells (10) were grown in folate free RPMI 1640 medium (Gibco, Life Technologies, Strathclyde, UK) supplemented with 10 % heat inactivated dialysed fetal calf serum (PAA laboratories, Yeovil, UK) and antibiotics in T75 tissue culture flasks at a seeding density of 0.5×10⁶ (5% CO₂ incubator; 37°C). Leucovorin (RS-5-formyl tetrahydrofolic acid) was added to the growth medium to a final concentration of 20nM to approximate the concentration of folates in human plasma. At ~60% confluence the cells were exposed to increasing concentrations (0.1 – 500 µg/ml) of BGC 9331 and BGC 945 in growth media for 2 hours. Drug-containing medium was removed, the cells were then washed and pulsed with [³H]Thd (0.37 MBq per flask) for 1h to determine whole cell and DNA associated radioactivity as previously reported (8). Data were quench-corrected and expressed as cpm/µg protein for whole cell and cpm/µg DNA for DNA.

Kinetics of nucleoside transport

Exponentially growing KB cells were treated for 2 h with BGC 9331 or BGC 945 at concentrations of 10 or 100 µg/ml or equal volume of vehicle (PBS). Single cells prepared by trypsinisation were kept suspended in culture medium by mild agitation with a shaker. Plasma membrane NBMPR-sensitive nucleoside transporter binding sites (a measure of membrane ENT1 (8)) on KB cells were measured by incubation of 2×10⁶ cells with 0.1, 0.25, 0.5, 1, 2, 4, 8, 16, 32 and 48 nM of [³H]NBMPR in medium. The total incubation volume was 400 µl and the studies were carried out at room temperature (~20°C) for 60 min (equilibrium binding). In parallel experiments to assess non-specific binding, equal numbers of cells were pre-incubated with 20 µM of the specific inhibitor, nitrobenzylthioguanosine, for 15 min and then incubated with [³H]NBMPR at the same concentration range as above. To analyse whether the increase in nucleoside transport was due to increases in cellular synthesis of the transporter protein, similar experiments were conducted on cells pre-treated for 15 min with cycloheximide (10 µg/ml). At the end of incubation, all reactions were quickly stopped and processed as previously reported (8). Specific binding (binding sites per cell) was defined as the difference in [³H]NBMPR content in the presence and absence of nitrobenzylthioguanosine. The maximal binding (B_{max}; the number of transporters) and the concentration of ligand required to reach half-maximal binding (K_D; affinity) were determined by curve fitting using GraphPad Prism, version 4 (GraphPad, San Diego, CA). Data were fitted to the equation: $Y = B_{am} \times X / (K_D + X)$, where Y and X are the specific binding and [³H]NBMPR concentration in nM, respectively. Experiments were performed in triplicate.

Animals, tumour model and drug treatment

The tumour model used in this study was the α-FR-positive KB xenograft grown in Balb/c nu/nu mice. For this procedure, KB tumour cells cultured as described above were harvested and resuspended in unsupplemented RPMI 1640 medium at 5×10⁷ cells/ml. Tumours were induced by subcutaneous injection of 5×10⁶ cells in 50 µl of the cell suspension into the back of 6-8 week-old female Balb/c nu/nu mice obtained from Harlan United Kingdom Ltd (Bicester, United Kingdom). Tumour dimensions were measured continuously using a calliper and tumour volumes were calculated by the equation: volume = (π/6) × a × b × c, where a, b, and c represent three orthogonal axes of the tumour. The tumours attained a size of about 100 mm³ at approximately 14 days later. Given that normal mouse chow is supplemented with high concentrations of folic acid and consequently mice have high

plasma folate levels (~ 5- to 10-fold higher than humans) that may reduce BGC 945 binding to FR, mice that were to be treated with BGC 945 were fed mouse chow unsupplemented with folic acid (supplied as pellets from TestDiet, Richmond, IL) starting 5 days before the inoculation of KB cells and then throughout the tumour growth and drug treatment phase until imaging studies were done in an attempt to reduce folate levels to that found in humans (11, 12). Since BGC 9331 is predominantly transported by the high capacity RFC, studies with this compound were performed under normal dietary conditions as has been reported by other investigators (5, 13, 14); to allow for direct comparison, imaging studies were also performed after vehicle or BGC 9331 injection (100 mg/kg; 4 h post treatment) in a separate cohort of mice fed chow unsupplemented with folic acid. Mice were treated with drug and/or used for imaging studies when their tumours reached approximately 100 mm³. For imaging studies, size-matched tumour bearing mice were randomised and administered a single 100mg/kg injection of BGC 9331 or BGC 945 intraperitoneally (i.p.) and imaged at different times after drug injection (vehicle, 1, 4, 24, and 48 h) to assess the time course of target modulation. Images were also acquired 4 hours after a range of different doses of each compound was administered (vehicle, 25, 50, 100, 150 and 200 mg/kg). All animal work was performed by licensed investigators in accordance with the UK's 'Guidance on the Operation of Animals (Scientific Procedures) Act 1986' (HMSO, London, UK, 1990) and in full compliance with government regulations and guidelines on the welfare of animals in experimental neoplasia (15).

PET studies

[¹⁸F]FLT-PET studies were carried out on a dedicated small animal PET scanner, quad-HIDAC (Oxford Positron Systems, Weston-on-the-Green, United Kingdom) (8, 16, 17). For scanning, the tail veins of vehicle-, BGC 9331- or BGC 945-treated mice were cannulated after induction of anaesthesia with isoflurane-O₂-N₂O mixture. The animals were placed within a thermostatically controlled jig and positioned prone in the scanner. The jig was calibrated to provide a rectal temperature of ~37°C. A bolus injection of [¹⁸F]FLT (2.96-3.7 MBq) was administered intravenously via the tail vein cannula and scanning commenced. Dynamic scans were acquired in list-mode format over a 60 min period without attenuation correction. The acquired data were sorted into 0.5-mm sinogram bins and 19 time frames (0.5 × 0.5 × 0.5 mm voxels; 4 × 15 s, 4 × 60 s, and 11 × 300 s) for image reconstruction, which was performed by filtered back-projection using a two-dimensional Hamming filter (cut-off 0.6). The image data-sets obtained were visualized using the Analyze software (version 6.0; Biomedical Imaging Resource, Mayo Clinic, Rochester, MN).

Image analysis and quantification of radiotracer uptake

Cumulative images comprising of 30-60 min of the dynamic data were used for visualisation of radiotracer uptake and to draw regions of interest (ROI). ROI (each 0.5 mm thickness) were defined on five slices of tumour, small intestine (as an example of a proliferative TS-responsive normal tissue) and heart (blood volume (17)). Dynamic data from these slices were averaged for each tissue and at each of the 19 time points to obtain time versus radioactivity curves (TACs) for these tissues. Tissue radioactivity was normalized to that of heart to obtain a normalised uptake value (NUV); The NUV at 60 min post injection (NUV₆₀) was used for comparisons. The area under the NUV curve (AUC) was calculated as the integral of NUV from 0-60 min. The fractional retention of tracer (FRT) at 60 min relative to that at 2.5 min was also calculated.

Analysis of tumour thymidine kinase 1 protein (TK1) and co-factor levels

TK1 protein and co-factor (ATP) levels were assessed by Western blot and bioluminescence assays, respectively. KB tumours were excised from a cohort of mice treated with PBS or 100 mg/kg of the drug at 2 h post injection and snap-frozen in liquid nitrogen. Tumours

were pulverized in liquid nitrogen and homogenized in ice cold Dulbecco's PBS using an Ultra-Thurrax homogenizer (IKA, Staufen, Germany). TK1 levels were then analysed by Western blot as previously described (8). ATP levels were determined by a bioluminescence assay as previously described (8) using the same supernatants used for the Western blots above.

Analysis of tumour dUrd levels

KB tumours were taken from mice immediately after the imaging studies, snap-frozen in liquid nitrogen and stored at -80°C until use. To confirm that thymidylate synthase was inhibited by BGC 9331 and BGC 945, dUrd levels were assessed by a chromatographic assay (7, 8). Briefly, whole (weighed) tumour samples were homogenized and the homogenate initially clarified by perchlorate protein precipitation; after neutralization and centrifugation the supernatant was further clarified by solid phase extraction. After evaporation to dryness using a vacuum centrifuge the samples were reconstituted for analysis by HPLC as previously described (7). dUrd was separated on a C18 stationary-phase with a mobile-phase comprising of 0.1% trifluoroacetic acid and detected at 267nm using a UV-photodiode-array detector. dUrd losses from sample preparation were estimated by measuring the recovered activity of $[5-^3\text{H}]$ -dUrd spiked into blank tumour samples. Chromatographic peak areas for the samples were compared to a calibration curve; data were expressed as nmol/g.

Statistical analysis

Statistical analyses were performed using the software GraphPad Prism, version 4 (GraphPad, San Diego, CA). Multiple data sets comprising of different time points and dose levels were compared using a One-Way Analysis of Variance; Dunnett's Multiple Comparison Test was used to analyse the significance of the different groups. Two-tailed P value 0.05 were considered significant.

RESULTS

BGC 9331 and BGC 945 induce cellular uptake of $[^3\text{H}]$ Thd

The two TS inhibitors, BGC 9331 and BGC 945 increased the whole cell uptake of $[^3\text{H}]$ Thd into KB cells, as well as its incorporation into the DNA in a concentration-dependent manner (Supplementary Figure 1a-d). The effect was highest at 250-500 $\mu\text{g/ml}$ (2-3-fold increase) in both whole cells and DNA (Supplementary Figure 1a-d). Under the low folate culture conditions used, both BGC 9331 and BGC 945 had similar potencies for inducing $[^3\text{H}]$ Thd uptake.

BGC 9331 and BGC 945 induce redistribution of ENT1 to plasma membrane

Because of a 1:1 stoichiometry between NBMPR and membrane ENT1 levels, the $[^3\text{H}]$ NBMPR assay permits the number and affinity of membrane associated ENT1 to be measured (18-20). With respect to the kinetics of $[^3\text{H}]$ NBMPR uptake in KB cells, a drug concentration-dependent increase in the specific uptake of $[^3\text{H}]$ NBMPR i.e., the difference between the uptake of $[^3\text{H}]$ NBMPR in the presence and absence of NBTG, was seen (Supplementary Figure 2a and 2b). A summary of the number of membrane ENT1 (B_{max}) and transporter affinity (K_D) are shown in Table 1. Both drug concentrations of BGC 9331 and BGC 945 significantly increased ENT1 B_{max} on KB cells; treatment of cells with 100 $\mu\text{g/ml}$ of BGC 9331 or BGC 945 led to a 2-fold increase in ENT1 B_{max} . Under the conditions used, both BGC 9331 and BGC 945 had similar potencies for increasing B_{max} . In contrast, no relevant changes in transporter affinity were observed; the single unit change in K_D with BGC 9331 at the higher drug concentration is unlikely to be biologically

significant. $B_{\max}$ and K_D were unchanged following pre-treatment of cells with cyclohexamide indicating that the effect was independent of intracellular protein synthesis.

BGC 945 selectively increases tumour but not intestine [^{18}F]FLT uptake in vivo

We used the Thd analogue, [^{18}F]FLT and PET to determine the in vivo activity of the two TS inhibitors. In keeping with the in vitro findings above, both BGC 9331 and BGC 945 (100 mg/kg body weight, i.p.) induced an increase in [^{18}F]FLT-derived radioactivity in KB xenografts compared to tumours in vehicle-treated mice (Figure 1a-d). Of interest to its selectivity for FR, BGC 945 did not induce an observable change in intestinal radiotracer uptake, whereas BGC 9331 induced radiotracer uptake in this TS responsive normal tissue at 1 and 4 h (Figure 1a-d).

Summary radiotracer pharmacokinetic parameters derived from quantitative analysis of BGC 9331-induced (100 mg/kg) time-dependent increases in tissue radioactivity are presented in Table 2a (see TAC's in Supplementary Figure 3a-c). Under the conditions of this study, BGC 9331 increased tumour AUC, NUV_{60} and FRT within 1 h of drug injection. AUC, NUV_{60} and FRT peaked at 1-4 h, returning to baseline levels at 24 and 48 h after drug treatment (Table 2a); comparative data obtained in small cohorts of mice fed chow unsupplemented with folic acid demonstrated longer duration of action (> 24h) under these lower folate conditions (data not shown). With BGC 9331, the radiotracer uptake in intestine mirrored that of tumour (Table 2). Overall brain tissue had much lower radiotracer uptake and no relevant changes in radiotracer accumulation were seen. Table 2b shows that the increase in AUC, NUV_{60} and FRT was dose-related in tumour, at the 4h timepoint; the effect was detectable at the first drug dose of 50 mg/kg reaching maximal levels at higher drug concentrations. The time course of radiotracer uptake into tumour as measured by [^{18}F]FLT-PET was slower after BGC 945 than with BGC 9331, peaking at 4-24 h and returning towards baseline at 48 h (Table 3a). In contrast to the radiotracer kinetics observed in tumour, there were no changes in AUC, NUV_{60} or FRT in intestine following treatment with 100 mg/kg BGC 945 demonstrating selectivity for tumour; the change in brain radioactivity at 24 h is unlikely to be biologically significant. The changes in AUC, NUV_{60} and FRT induced by BGC 945 were detectable at a low dose of 25 mg/kg and showed a dose-related increase at 100 mg/kg (Table 3b). Unexpectedly, there was a reduction in the AUC and NUV_{60} parameters at 200 mg/kg compared to that at 100 mg/kg. FRT remained high at this dose. Also of interest, at the dose of 200 mg/kg, a detectable increase in intestinal radiotracer uptake was observed although this did not reach statistical significance ($p = 0.06$ for all imaging parameters; Table 3b).

TS-induced increase in [^{18}F]FLT uptake is not due to an increase in TK1 protein

To rule out increases in TK1/ATP being a contributing mechanism to the increase in [^{18}F]FLT uptake after TS inhibition and provide a clearer understanding of the time-dependent profile of antifolate drug effect, we assessed TK1 enzyme and ATP levels after BGC 9331 treatment in a cohort of mice (Figure 2a-c). Rather than an increase, TK1 enzyme levels were unchanged within 4 h of drug treatment and decreased significantly at 24 h ($p = 0.035$) returning to baseline levels at 48 h (Figure 2b). ATP levels were also maintained for 4 h but significantly decreased at 24 and 48 h (Figure 2c; $p = 0.03$).

Increases in [^{18}F]FLT uptake are associated with increases in tumour dUrd levels

The drug-induced increase in [^{18}F]FLT accumulation reported above were associated with increases in tumour dUrd levels (Figure 3a-c). Tumour dUrd levels increased by 10-13-fold at the peak time of 1-4 h after a 100 mg/kg BGC 9331 injection, returning to approximately control levels at 24 and 48h (Figure 3a). The increase in dUrd levels after BGC 9331 was not clearly dose related (Figure 3b). In keeping with the PET imaging studies, a small cohort

of BGC 9331-treated mice fed chow unsupplemented with folic acid showed similar high tumour dUrd levels at 4 and 24 h (data not shown). A >10-fold increase in tumour dUrd was also seen after 100mg/kg BGC 945 and this increase persisted for at least 24 hours (Figure 3c).

DISCUSSIONS

We have demonstrated by [^{18}F]FLT-PET imaging that BGC 945 selectively inhibits TS in the α -FR positive KB tumour but not in normal proliferating tissues at doses up to 200mg/kg. Because TS, RFC, and other relevant targets of antifolate pharmacology are ubiquitously expressed in both tumour and normal proliferating tissues, antifolates rarely display high selectivity for tumours over normal tissues. The antifolate BGC 945, however, was designed to be transported into cells by the FR (11), which unlike the RFC, is highly expressed on a number of tumours coupled with a restricted functional expression on the apical surfaces of some normal tissues (3, 4). This implies that unlike classical antifolates (such as BGC 9331) that can be used to treat several tumour types, the utility of BGC 945 will be restricted to tumours expressing the FR. On the other hand, we will expect the therapeutic index with BGC 945 to be high as BGC 945 potentially inhibits the growth of FR expressing cells under physiological folate conditions (11). Antifolates like BGC 9331 also demonstrate high TS and growth inhibitory potencies in both FR and non-FR expressing cells. In this study, we sought to demonstrate relative selectivity of TS inhibition by BGC 945 in tumours over normal tissues using a clinically translatable imaging method.

We previously demonstrated that TS inhibition is amenable to non-invasive imaging (8, 21). In the present report, we initially demonstrated that both the relatively non-selective (BGC 9331) and the α -FR-selective (BGC 945) TS inhibitors, possessed approximately equivalent intrinsic cellular potencies to increase nucleoside transport in the α -FR expressing human epidermoid KB tumour cells cultured under approximately physiological folate conditions in a dose dependent manner. We showed that [^3H]Thd uptake into whole cell and incorporation into DNA were enhanced by antifolate treatment. As with our previous studies with 5-FU (8), we confirmed that for both drugs the mechanism for enhanced nucleoside uptake could be explained at least in part by ~ 2 -fold induction of ENT1 redistribution to the cell membrane. The process was not inhibited by cycloheximide and hence could not be attributed to increased ENT1 protein synthesis nor could it be attributed to increased expression of TK1 protein and ATP, as levels of these did not increase in vivo. The data could be further supported in the future by directly measuring TK1 enzyme activity levels.

As a prelude to clinical studies, we wanted to know if [^{18}F]FLT uptake increased with BGC 945 treatment and to determine an optimal imaging time for early clinical trials of the drug. With regard to developing appropriate/predictive tumour models, there are several challenges in the pre-clinical development of targeted antifolates like BGC 945. The first challenge is related to the ~ 5 -fold higher plasma folate concentration in mice compared to humans (11, 12). To avoid competitive inhibition of BGC 945 binding to FR on KB tumours by folate, the mice were fed a diet unsupplemented with folic acid so as to achieve folate levels approximately that found in humans. Mice also have ~ 100 -fold higher Thd levels than humans (6). The high Thd on one hand can reduce the overall uptake of the radiotracer, [^{18}F]FLT, in this species (22). We have, however, been able to demonstrate utility of the radiotracer for monitoring TS inhibition in mice despite the systematic reduction in absolute radiotracer uptake (8); this works in favour of humans because of their lower Thd levels (6, 23). Of relevance to the efficacy of TS inhibitors, the high Thd levels can bypass the effects of TS inhibition through the activity of TK1 making these models less predictive of human efficacy (12). The use of a TK-deficient tumour model can overcome this limitation. For example, treatment with BGC 9331 has been shown to be exquisitely efficacious in the TK-

deficient mouse lymphoma tumour model, L5178Y TK^{-/-} at doses of 10 mg/kg due to the inability of this tumour to salvage Thd (14). The same model cannot be used to study efficacy of BGC 945, however, due to its lack of FR expression. Furthermore, even with the L5178Y TK^{-/-} model, therapeutic index cannot be easily assessed as dThd salvage is still in operation in normal tissues. Because of these challenges in transferring knowledge of pre-clinical efficacy of antifolates into clinical development, we decided to compare our imaging readout to another biomarker of TS inhibitory activity, dUrd.

Inhibition of TS leads to an increase in dUMP and dUrd in proliferating cells (24) and an increase in dUrd can be detected in mice and in humans after administration of TS inhibitors (4, 5). Plasma dUrd is therefore a useful PD marker of TS inhibition in proliferating tissue such as gut, bone-marrow and tumour. We have adapted the assay to specifically measure tumour dUrd, to assess localised TS inhibition. For both BGC 9331 and BGC 945 tumour dUrd increased ~10-fold in a time-dependent fashion. This effect was not dose-related at 4h following BGC 9331 injection, consistent with our previous experience (unpublished data). This may be due to the dynamics of dUrd in tissues and plasma – efflux mechanisms, feedback loops and systemic elimination. Since we did not directly measure TS inhibition but rather changes in dUrd levels as a surrogate for TS inhibition, the lowest level of TS inhibition that shows an increase in FLT uptake is unknown. Dose-dependence following BGC 945 was not assessed in this present study. BGC 9331 at 100 mg/kg induced an increased radiotracer accumulation in both tumours and small intestines as shown by [¹⁸F]FLT-PET and this effect was dose-related. NUV₆₀, AUC and FRT all peaked at 1-4 h and returned towards baseline at 24 h demonstrating rapid onset and relatively short duration of action. This short duration of action was expected, as BGC 9331 does not undergo polyglutamation and therefore has rapid elimination in mice. In contrast, at an equivalent dose of 100 mg/kg, BGC 945 induced increased radiotracer accumulation in tumour but not intestine demonstrating tissue specificity. This tissue specificity was not due to the different dietary conditions used since mice on chow unsupplemented with folic acid treated with BGC 9331 showed similar increased radiotracer uptake in intestine (data not shown). For BGC 945, NUV₆₀, AUC and FRT peaked at 4-24 h and returned towards baseline at 48 h. Gibbs et al have reported that BGC 945 is rapidly cleared from mouse plasma but retained in KB tumours with a terminal half life of 28 h (11). Provided that the pharmacokinetics of BGC 945 in humans is such as to permit sufficient drug accumulation in tumours, ~24 h will be the ideal time for imaging FR-mediated TS inhibition in humans with the PET technology.

Given that toxicities from antifolates (25) including myelosuppression and diarrhoea are related to their effects on normal proliferating tissue, our PET findings for BGC 945-treated mice are strongly indicative of a high (biomarker-based) therapeutic index in subjects with FR-positive tumours. [¹⁸F]FLT-PET may have utility for predicting activity in tumours or sub-clinical normal tissue toxicity. Drug doses that induce measurable [¹⁸F]FLT uptake may be used to define the BED. On the other hand, a detectable but non-significant increase in intestinal [¹⁸F]FLT accumulation was observed with BGC 945 at 200 mg/kg that may be attributed to transient TS inhibition in normal proliferating tissue induced by non-FR uptake when drug levels are high; this can be used as a basis to define sub-clinical toxicity (although the exact relationship between the extent of TS inhibition in normal tissues and gastrointestinal toxicity is unknown and will need to be assessed clinically). With respect to imaging parameters, we found that NUV₆₀, AUC and FRT were useful parameters for assessing TS inhibition at and below 100 mg/kg of BGC 945. The reduction in tumour NUV₆₀ and AUC at 200 mg/kg was unexpected and inconsistent with the in vitro cell culture [³H]Thd studies where high doses led to a plateau in radiotracer uptake. We speculate that the reduction in NUV₆₀ and AUC at this very high dose is due to non-specific effect of the drug on radiotracer delivery. This assertion is supported by the absence of a

reduction in the FRT parameter. FRT, a parameter that describes the fraction of radiotracer reaching a tissue that is retained, was still high at 200 mg/kg dose indicating utility of this parameter at such high doses. Of note, only single dose pharmacodynamic changes were assessed in this report. The technology may have limitations when used after repeat dosing with antifolate TS inhibitors as decreases in [^{18}F]FLT accumulation reflecting a reduction in proliferation and hence TK1 enzyme activity can occur under such conditions (16, 17, 21).

In summary, we have used [^{18}F]FLT-PET imaging to demonstrate tumour specific TS inhibition by BGC 945 and shown that a likely mechanism by which [^{18}F]FLT-PET measures TS inhibition involves redistribution of nucleoside transporters to the cell membrane. Depending on the pharmacokinetics of BGC 945 in humans, clinical studies should consider a protocol involving a baseline [^{18}F]FLT-PET scan followed by another [^{18}F]FLT-PET scan in the same patient at ~24 h after drug treatment when peak effects are seen.

SUPPLEMENTARY MATERIAL

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Typical 0.5 mm coronal $[^{18}\text{F}]$ FLT-PET slices through the ventral (showing intestine and bladder) and dorsal regions (showing KB tumour) of control (a,c), 100 mg/kg BGC 9331 (b), and 100 mg/kg BGC 945-treated mice (d) imaged at 4 h post-treatment. The white and yellow arrow-heads point to tumour and intestine, respectively, and bladder is seen as a high intensity body in the caudal region of the mice. All images shown here were obtained from mice fed chow unsupplemented with folic acid. Note colour bar.

Figure 2.

a. Typical example of TK1 and β -actin (loading control) protein levels determined by Western blot at various time-points after vehicle or 100 mg/kg BGC 945 treatment as described in the Materials and Methods section. **b.** Summary data for TK1, which were expressed as ratios of the optical density of TK1 to β -actin bands. **c.** Summary data for ATP determined with an in vitro bioluminescence kit as described in the Materials and Methods section and expressed as a percentage of vehicle control. Data are mean $\pm$ SD (N = 4 repeats). *, P < 0.05.

Figure 3.

Deoxyuridine (dUrd) levels in mouse KB tumours after treatment with BGC 9331 or BGC 945. **a.** BGC 9331 time-dependence (standard chow), **b.** BGC 9331 dose-dependence (standard chow), **c.** BGC 945 time-dependence (chow unsupplemented with folic acid). The levels of dUrd were measured by HPLC as described in the Materials and Methods section. Data are individual mouse tumour dUrd levels and mean (solid line). *, $P < 0.05$.

Table 1
Effect of BGC 9331 and BGC 945 on ENT1 redistribution to the membrane of KB cells in vitro.

Data are mean $\pm$ SD.

One-way ANOVA (all groups; as superscript on parameters) and multiple comparison test against control values (Dunnett; as superscript on individual values):

* p 0.05

** p 0.01

*** p 0.001.

		Control	10 μ g/ml	100 μ g/ml
		N=3	N=3	N=3
BGC 9331	B _{MAX} (number of binding sites/cell)***	55720 $\pm$ 6101	90430 $\pm$ 6384**	118700 $\pm$ 5193**
	K _D (nM)*	5.8 $\pm$ 0.4	6.3 $\pm$ 0.9	7.7 $\pm$ 0.3*
BGC 945	B _{MAX} (number of binding sites/cell)***	55720 $\pm$ 6101	95840 $\pm$ 1340**	130800 $\pm$ 10800**
	K _D (nM)	5.8 $\pm$ 0.4	7.1 $\pm$ 0.3	7.4 $\pm$ 3.0

Table 2

a) Time course and b) dose response studies with BGC 9331. A summary of [¹⁸F]FLT-PET pharmacokinetic parameters in control and BGC 9331-treated KB tumour bearing mice. Imaging studies were performed at different time points after mice (on standard diet) were treated with a single (100mg/Kg) dose of the drug (time course studies) or at 4 h after mice were injected with different doses of the drug (dose response studies).

Data are mean ± SD

One-way ANOVA (all groups; as superscript on parameters) and multiple comparison test against control values (Dunnett; as superscript on individual values):

* p 0.05

** p 0.01

*** p 0.001.

a). Time course									
Tissue	Control n = 4	1h n = 3		4h n = 5		24h n = 3		48h n = 3	
		Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
Tumour	AUC***	55 ± 9.2	109 ± 11**	119 ± 2.8**	42 ± 5.4	60 ± 7.9			
	NUV60***	1.2 ± 0.22	3.0 ± 0.36**	3.4 ± 0.12**	1.0 ± 0.17	1.5 ± 0.13			
	FRT60/2.5***	3.3 ± 0.18	7.7 ± 0.60**	8.2 ± 1.1**	4.3 ± 0.86	6.6 ± 2.8*			
Small Intestine	AUC***	60 ± 10	102 ± 5.2**	101 ± 17**	67 ± 6.1	66 ± 4.2			
	NUV60***	1.2 ± 0.18	2.6 ± 0.16**	2.5 ± 0.52**	1.3 ± 0.15	1.2 ± 0.11			
	FRT60/2.5**	1.8 ± 0.30	4.4 ± 2.3**	3.5 ± 0.54*	1.5 ± 0.22	1.4 ± 0.23			
Brain	AUC	13 ± 0.61	16 ± 0.90	15 ± 2.4	13 ± 2.6	13 ± 1.6			
	NUV60	0.25 ± 0.027	0.32 ± 0.028	0.30 ± 0.064	0.28 ± 0.041	0.26 ± 0.017			
	FRT60/2.5*	1.7 ± 0.29	2.4 ± 0.46*	2.1 ± 0.16	2.0 ± 0.26	2.2 ± 0.17			
b). Dose response									
Tissue	Control n = 4	50mg/kg n = 6		100mg/kg n = 6		150mg/kg n = 3		200mg/kg n = 3	
		Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
Tumour	AUC***	55 ± 9.2	91 ± 9.6**	119 ± 2.8**	120 ± 33**	146 ± 8.8**			
	NUV60***	1.2 ± 0.22	2.4 ± 0.41**	3.4 ± 0.12**	3.6 ± 0.95**	4.4 ± 0.32**			
	FRT60/2.5***	3.3 ± 0.18	6.9 ± 2.6*	8.2 ± 1.1**	9.2 ± 1.1**	8.9 ± 0.65**			
Small Intestine	AUC**	60 ± 10	84 ± 20	101 ± 17**	100 ± 1.9*	101 ± 6.6**			

a). Time course		Control n = 4	1h n = 3	4h n = 5	24h n = 3	48h n = 3
Tissue		Mean $\pm$ SD	Mean $\pm$ SD	Mean $\pm$ SD	Mean $\pm$ SD	Mean $\pm$ SD
		NUV60**	1.2 $\pm$ 0.18	1.8 $\pm$ 0.76	2.5 $\pm$ 0.52**	2.0 $\pm$ 0.48
		FRT60/2.5**	1.8 $\pm$ 0.30	2.1 $\pm$ 1.1	3.2 $\pm$ 0.25	2.5 $\pm$ 0.59
Brain		AUC*	13 $\pm$ 0.61	14 $\pm$ 1.5	15 $\pm$ 2.4	18 $\pm$ 1.6**
		NUV60	0.25 $\pm$ 0.027	0.33 $\pm$ 0.084	0.30 $\pm$ 0.064	0.38 $\pm$ 0.0092
		FRT60/2.5	1.7 $\pm$ 0.29	2.6 $\pm$ 0.72	2.1 $\pm$ 0.16	2.0 $\pm$ 0.43

Table 3

a). Time course and b). dynamic range studies with BGC 945. A summary of [¹⁸F]FLT-PET pharmacokinetic parameters in control and BGC 945-treated KB tumour bearing mice. Imaging studies were performed at different time points after mice (on folate-deficient diet) were treated with a single (100mg/Kg) dose of the drug (time course studies) or at 4 h after mice were injected with different doses of the drug (dynamic range studies).

Data are mean $\pm$ SD

One-way ANOVA (all groups; as superscript on parameters) and multiple comparison test against control values (Dunnett; as superscript on individual values):

* p 0.05

** p 0.01

*** p 0.001.

a). Time course					
Organ		Control n = 8	1h n = 3	4h n = 3	24h n = 4
		Mean $\pm$ SD	Mean $\pm$ SD	Mean $\pm$ SD	Mean $\pm$ SD
Tumour	AUC***	57 $\pm$ 7.7	101 $\pm$ 21**	116 $\pm$ 22**	124 $\pm$ 26**
	NUV60***	1.3 $\pm$ 0.23	2.8 $\pm$ 0.73*	3.2 $\pm$ 0.77**	3.5 $\pm$ 0.90**
	FRT60/2.5	4.4 $\pm$ 2.0	7.5 $\pm$ 1.8	6.7 $\pm$ 0.52	8.4 $\pm$ 4.0
Small Intestine	AUC	66 $\pm$ 6.4	62 $\pm$ 6.8	69 $\pm$ 19	74 $\pm$ 12
	NUV60	1.2 $\pm$ 0.14	1.1 $\pm$ 0.17	1.3 $\pm$ 0.51	1.3 $\pm$ 0.21
	FRT60/2.5	1.2 $\pm$ 0.30	1.2 $\pm$ 0.22	1.4 $\pm$ 0.40	1.2 $\pm$ 0.37
Brain	AUC*	15 $\pm$ 1.9	15 $\pm$ 2.1	16 $\pm$ 1.2	19 $\pm$ 2.0*
	NUV60	0.29 $\pm$ 0.046	0.28 $\pm$ 0.046	0.30 $\pm$ 0.041	0.37 $\pm$ 0.055
	FRT60/2.5	1.9 $\pm$ 0.41	1.9 $\pm$ 0.21	1.8 $\pm$ 0.30	1.9 $\pm$ 0.36
b). Dynamic range					
Tissue		Control n = 8	25mg/kg n = 3	100mg/kg n = 3	200mg/kg n = 3
		Mean $\pm$ SD	Mean $\pm$ SD	Mean $\pm$ SD	Mean $\pm$ SD
Tumour	AUC**	57 $\pm$ 7.7	67 $\pm$ 26	116 $\pm$ 22**	62 $\pm$ 24
	NUV60***	1.3 $\pm$ 0.23	1.8 $\pm$ 0.75	3.2 $\pm$ 0.77**	1.8 $\pm$ 0.86
	FRT60/2.5	4.4 $\pm$ 2.0	5.3 $\pm$ 2.4	6.7 $\pm$ 0.52	8.3 $\pm$ 5.8
Small Intestine	AUC	66 $\pm$ 6.4	67 $\pm$ 11	69 $\pm$ 19	83 $\pm$ 15

a). Time course						
Organ	Control n = 8	1h n = 3	4h n = 3	24h n = 4	48h n = 4	
		Mean $\pm$ SD	Mean $\pm$ SD	Mean $\pm$ SD	Mean $\pm$ SD	
Brain	NUV60	1.2 $\pm$ 0.14	1.1 $\pm$ 0.088	1.3 $\pm$ 0.51	1.7 $\pm$ 0.47	
	FRT60/2.5	1.2 $\pm$ 0.30	1.0 $\pm$ 0.17	1.4 $\pm$ 0.40	2.4 $\pm$ 1.4	
	AUC	15 $\pm$ 1.9	16 $\pm$ 2.6	16 $\pm$ 1.2	15 $\pm$ 1.7	
	NUV60	0.29 $\pm$ 0.046	0.33 $\pm$ 0.045	0.30 $\pm$ 0.041	0.31 $\pm$ 0.024	
	FRT60/2.5	1.9 $\pm$ 0.41	2.3 $\pm$ 0.16	1.8 $\pm$ 0.30	2.3 $\pm$ 0.41	