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Increased tumour extracellular pH induced by Bafilomycin A_1 inhibits tumour growth and mitosis *in vivo* and alters 5-fluorouracil pharmacokinetics

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Abstract

The aim was to determine if a specific inhibitor of vacuolar H^+-ATPases (V-ATPases), Bafilomycin A_1 (BFM), could increase the low extracellular pH (pHe) typical of solid tumours and thus inhibit their growth in vivo. BFM inhibited the proliferation of various human cells and rat pituitary GH3 tumour cells *in vitro* (IC_{50}: 2.5–19.2 nM), and flow cytometry on GH3 cells showed a marked increase in S and G2M phases after 16–48 h, but no evidence of increased apoptosis. BFM caused significant inhibition of GH3 xenograft growth, and histomorphometry showed a 30% decrease in mitosis but no change in apoptosis. ^{31}P-magnetic resonance spectroscopy (MRS) *in vivo* of GH3 xenografts showed that BFM increased pHe, but did not affect pHi, resulting in a decrease in the negative pH gradient (-ΔpH). BFM decreased lactate formation suggesting a reduction in glycolysis. We suggest that BFM reduces extracellular H^+-transport by inhibition of V-ATPases leading to an increase in pHe and decreased glycolysis, and thus reduced tumour cell proliferation. ^{19}F-MRS *in vivo* showed that a smaller -ΔpH was associated with decreased retention of 5-fluorouracil (5FU) which was consistent with our previous data *in vivo* implying the -ΔpH controls tumour retention of 5FU.

Keywords: Bafilomycin; pH; ^{31}P-MRS; 5FU uptake; Solid tumours

1. Introduction

Maintenance of intracellular pH (pHi) is crucial to cell viability, and in mammalian cells it is tightly regulated by the concerted action of a number of different carriers and pumps in the plasma membrane [reviewed in 1]. Magnetic resonance spectroscopy (MRS) has shown that the pHi of solid tumours is neutral or slightly alkaline compared with normal tissues (pH 7.0–7.4) and that the extracellular pH (pHe) is acidic [2,3]. The reasons for the acidic pHe are complex, but in part it is caused by the high glycolytic rate of tumour cells leading to increased export of H^+ from the intracellular compartment [4]. The relatively high pHi and low pHe lead to a so-called negative pH gradient (-ΔpH); the opposite of that found in normal cells [5]. These altered pH states have been considered to confer survival and growth advantages for solid tumour cells [5,6], and indeed, studies *in vitro* have shown that a low pHe favours metastatic behaviour and production of angiogenic factors by tumour cells [7,8]. On the other hand, the -ΔpH can also provide an opportunity for selective therapy, since drugs that behave as weak acids and enter cells by passive diffusion will be retained more by tumours than normal tissues [9]. We hypothesised that a drug causing a chronic increase in tumour pHe could lead to a slower growth of solid tumours *in vivo* and would impact in a

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predictable manner on tumour uptake of the anti-cancer drug 5-fluorouracil (5FU).

The drug we investigated was the macrolide antibiotic Bafilomycin A_1 (BFM). BFM is an inhibitor of cellular ATPase enzymes with a >10 000-fold specificity for vacuolar H^+-ATPases (V-ATPases) [10]. V-ATPases cause acidification of intracellular organelles, and are also involved in renal acidification, osteoclast-mediated bone resorption and regulation of macrophage pHi [11]. V-ATPases may be functionally expressed in the plasma membrane of some tumour cells since (a) BFM decreases the pHi of isolated tumour cells, particularly those with a high pHi, [6] and (b) over-expression of V-ATPases increases pHi *in vitro* [12]. A number of studies have shown that BFM can inhibit the proliferation of tumour cells in culture in a dose-dependent manner [13–16], with more transformed cells showing increased sensitivity [13]. V-ATPases are also involved in multi-drug resistance [17,18] and cisplatin resistance [12] and overexpression in human pancreatic cells has been associated with increased metastasis *in vivo* [19].

We have studied the effect of BFM on a number of tumour cell lines in culture and *in vivo* on a rat pituitary cell line, GH3, grown in nude mice and rats. GH3 tumours have a relatively high pHi *in vivo* as measured by ^{31}P-MRS and were therefore expectedx to be more sensitive to BFM *in vivo* [6]. The GH3 xenografts were studied non-invasively by ^{31}P-MRS and ^{19}F-MRS to investigate the effect of BFM on tumour pH and pharmacokinetics of the anticancer drug 5FU, respectively. The 5FU pharmacokinetic data were then compared with earlier published data which together suggested that alterations in the tumour transmembrane -ΔpH strongly impact on tumour retention of 5FU. The clinical significance of this is discussed.

2. Materials and methods

Bafilomycin A_1 (BFM) was kindly supplied by Glaxo-SmithKline (Welyn, UK) following isolation from *Streptomyces griseus* as previously described in Ref. [20]. 3-aminopropyl phosphonate (3-APP), 5FU and dimethyl sulphoxide (DMSO) were purchased from Sigma Chemicals Company Ltd, Poole, UK. Cell culture materials were obtained from Gibco, Paisley, UK. Hypnorm was obtained from Jansen Pharmaceuticals (UK) and Hypnovel from Roche (UK).

2.1. Cell culture

The rat prolactinoma cell line, GH3, was grown in house as previously described in Ref. [21] and the cell lines HOS (human osteosarcoma), HT29 (human colon adenocarcinoma) and human ovarian lines CH1 and CH1-Dox (*p*-glycoprotein expressing) [22,23] were obtained from the European Collection of Cell Cultures (ECACC) and the American Tissue Culture Collection (ATCC) and grown in standard cell culture medium including 10% fetal calf serum (FCS) at 37 °C in a 5% CO_2 atmosphere. For cytotoxic assays, cell number was determined using either the dimethylthiazolyl-2,5-diphenyl tetrazolium bromide (MTT) assay [24] or the sulphorhodamine B (SRB) assay [25]. The concentration that caused 50% inhibition (IC_{50}) was determined from a 3-parameter sigmoid plot of the data using Sigmaplot Software (SPSS Science Software, Birmingham, UK). For monoparametric cell cycle analysis, cells were fixed in 70% (v/v) ethanol (30 min on ice) and stained using propidium iodide (400 μg/ml for 30 min) and examined using a Coulter Counter flow cytometer (Beckman Coulter, Bucks, UK) equipped with an argon-ion laser (Spectra Physics, San Jose, CA) with an output of 200 mW at 488 nm as previously described in Ref. [26]. Typically, data from 2×10^4 cells were analysed for forward and orthogonally scattered light together with red fluorescence (peak and integrated area). Pulse shape analysis was performed to eliminate any cell clumps, and data were gated on light scatter before recording a histogram of red fluorescence using the WinMDI2.8 program (http://www.uwcm.ac.uk/uwcm/hg/hoy/index.html).

2.2. Tumours

GH3 prolactinoma cells (10^7) were injected subcutaneously (s.c.) in the flanks of Wistar-Furth rats (200 g) or MF1 nude mice (25 g) and maintained in the St. George's Hospital Medical School Biological Research facilities. Four different cohorts of animals were used: (1 and 2) tumours in rats and xenografted in nude mice for measurement of lactate (3) mouse xenografts for ^{31}P and ^{19}F-MRS analysis (4) mouse xenografts for growth inhibition and subsequent histological analyses. Tumour size (mm^3) was determined by measuring three orthogonal dimensions (d_1, d_2 and d_3) in mm and using the formula $(\pi/6)d_1d_2d_3$, and were used for lactate and MRS studies between 300 and 1000 mm^3 (see below). For growth inhibition studies, tumour-bearing nude mice were divided into two groups of equal mean size (>100 mm^3) and treatment with BFM (1 mg/kg intraperitoneally (i.p.)) or vehicle was begun 1–2 times weekly for 3–5 weeks. BFM was prepared on the day of use by dissolving BFM in 100% DMSO (1 mg/ml) and then diluting 1:1 in fresh distilled water to provide a final concentration of 0.5 mg/ml. For vehicle (control) treatment, 100% DMSO was diluted 1:1 in fresh distilled water on the day of use and injected at 0.5 ml/kg. Tumour size and animal weight was recorded every 2–3 days. At the end of the experiment, animals were killed by cervical dislocation, tumours excised and weighed and fixed in 70% (v/v) formalin. In one experiment, sections were stained using haematoxylin and eosin and

the percentage of tissue that was cellular, necrotic or vascular assessed using the Chalkley method as previously described in Ref. [3]. The mitotic and apoptotic index was determined at 600x magnification using light microscopy on at least 2000 cells for each tumour as previously described in Ref. [27].

2.3. Non-invasive MRS studies

Mice were anaesthetised as previously described in Ref. [3], using a single injection of a Hypnovel-Hypnorm mixture at least 30 min prior to all data acquisition. Prior to and 48 h after treatment with BFM (1 mg/kg) or vehicle, the mice were placed in the centre of a 4.7T Varian 200-330 spectrometer and body temperature maintained with a water-heated pad. Following injection of 3-APP for measurement of pHe, ^{31}P experiments were carried out using a 10 mm surface coil and image-guided localised spectroscopy by Image Selected In vivo Spectroscopy (ISIS) to minimise signal contamination from the underlying tissue [3]. ^{19}F-MRS was performed following the ^{31}P-MRS on mice treated 48 hr earlier by BFM (1 mg/kg) or vehicle, using an 18 mm surface coil. Spectra were acquired for 100 min immediately following injection of 5FU (130 mg/kg i.p.) as previously described in Ref. [28].

2.4. Tumour extracts

Tumours were freeze-clamped 48 h after treatment with BFM (1 mg/kg) or vehicle and perchlorate extracts made as previously described in Ref. [29]. Total lactate (intra- plus extracellular) was determined as described by Bergmeyer in Ref. [30].

2.5. Data analysis

^{31}P-spectra were analysed by VARPRO to determine peak integrals for nucleoside tri-phosphates (NTP), 3-APP, phosphate (Pi) and phosphomono- and diesters (PME and PDE) as well as chemical shifts for determination of pHe and pHi [3]. Data were expressed as NTP/Pi or NTP/TP (total phosphate), and PME/TP and PDE/TP. ^{19}F-spectra were analysed by the Varian spectrometer spectral fitting programme, FITSPEC, to determine peak integrals for 5FU and the 5-fluoronucleotides (FNuct). The half-time ($t_{1/2}$) for the rate of elimination of 5FU from each of the tumours was determined from the line-slope of a semi-log plot of the data, where the $t_{1/2} = \log(2)/\text{slope}$ [31]. Comparisons of tumour growth in vehicle and BFM-treated mice were assessed in three different ways using; (a) mean tumour sizes at various time points to provide a %T/C i.e. delta tumour volume of treated group divided by delta tumour volume of control group; (b) slopes during exponential growth to obtain the tumour doubling time (TDT); (c) time to reach 10% body weight following initiation of treatment. All data shows mean standard error of the mean ±(SEM) and significant differences between means were determined using Student's *t*-test or where appropriate a paired *t*-test, and $P < 0.05$ was considered significant.

3. Results

3.1. Effects of BFM and BFM-derivatives on cultured tumour cells

BFM inhibited cell proliferation in a dose-dependent manner, and for the five cell lines tested yielded mean IC_{50}'s between 3 and 20 nM (Table 1). For the human ovarian cell line, CH1, there was no difference in the IC_{50} between wild-type and the CH1-Dox cell line which overexpresses *p*-glycoprotein (CH1-Dox). Cell cycle analysis showed that GH3 cells incubated with 8 or 20 nM BFM showed a gradual decrease in the proportion of cells in G1-phase from 5 to 24 h as the S and G2M phases increased by 1.5 and 4-fold, respectively (Fig. 1). At 48 h, this was beginning to be reversed. A sub-G1-peak was not detected suggesting apoptosis was not induced by BFM under these conditions.

3.2. Effect of BFM on the growth of GH3 xenografts

A pilot study (data not shown) indicated that weekly injections of BFM had no significant effect on tumour growth, in terms of rate or the final ablated tumour volumes (2200±447 mm^3 versus 1480±402 mm^3, $P = 0.1$). A follow-up experiment using injections of BFM twice weekly (1 mg/kg) for up to 20 days caused a significant decrease in tumour size compared with controls after 10 and 13 days treatment giving T/C of 36 and 46%, respectively, and a 30% decrease in the average rate of growth (TDT increased from 7.9±0.9 to 11.2±0.9 days, $P = 0.018$). Following initiation of treatment, the time taken for the BFM-treated tumours compared with controls to reach 10% of mouse body

Table 1
Inhibition of tumour cell proliferation by BFM

Cell line	48 h (MTT)	96 h (SRB)
GH3	13.7±2.6 (2)	3.9 (1)
HOS	19.2±29.6 (4)	nd
HT29	8.4±1.1 (3)	2.5 (1)
CH1	nd	5.8±1.5 (2)
CH1-Dox	nd	5.7±3.6 (2)

SD, standard deviation; IC50, concentration causing 50% inhibition; MTT, dimethylthiazolyl-2,5-diphenyl tetrazolium bromide; SRB, sulphorhodamine B; BFM, Bafilomycin A. Data shows the mean IC_{50}±S.D. in nM from (*n*) experiments determined using the MTT or SRB assay (see Methods), nd, not done.

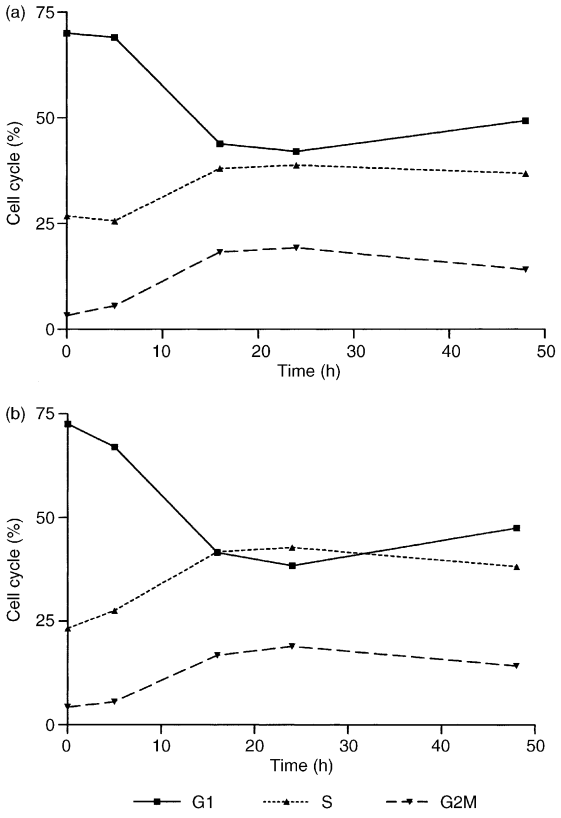

Fig. 1. Changes in cell cycle of GH3 cells induced by BFM. GH3 cells in exponential growth were incubated with Bafilomycin A (BFM) at a concentration of 8 nM (a) or 20 nM (b). Cells were fixed in 70% (v/v) ethanol at 5, 16, 24 or 48 h after BFM incubation and cell cycle analysis performed as described in Methods. Data shows mean of duplicate determinations for each time point.

weight was delayed by approximately 60% (21 days compared with 13 day) (Fig. 2). Mouse body weight was unaffected by either treatment and there were no other visible signs of toxicity. Morphological analysis of the ablated, fixed-tumours showed that approximately 1% of the cells were apoptotic and this was not significantly affected by BFM (Table 2). However, BFM significantly lowered the mitotic index by 30% ($P=0.004$) and the mitotic:apoptotic ratio was reduced by approximately 40% compared with controls ($P=0.005$). No significant differences were detected in the vascular or necrotic status of the tumours.

3.3. ^{31}P-MRS studies in vivo of effects of BFM on GH3 xenografts

^{31}P-MRS spectra showed that prior to BFM treatment, GH3 tumours (0.3–1.0 g) had mean pHi and pHe of 7.10 ± 0.02 and 6.95 ± 0.04, respectively ($\pm$SEM, $n=6$), providing a higher pHi than that recorded by this group for other tumours grown in mice (3). 48 hr after treatment with BFM, there was a significant increase in the pHe, while pHi was unchanged resulting in a significant decrease in the -ΔpH of 0.11 (Fig. 3). In contrast, vehicle-treated animals (controls) showed a decrease in pHe, while pHi was unchanged, resulting in an increase in the -ΔpH of 0.14 (Fig. 3). Although the mean changes in pHi and pHe for the controls were not significant, 6 of the tumours showed an increase in the -ΔpH which was significant by a paired t-test ($P<0.05$). Neither

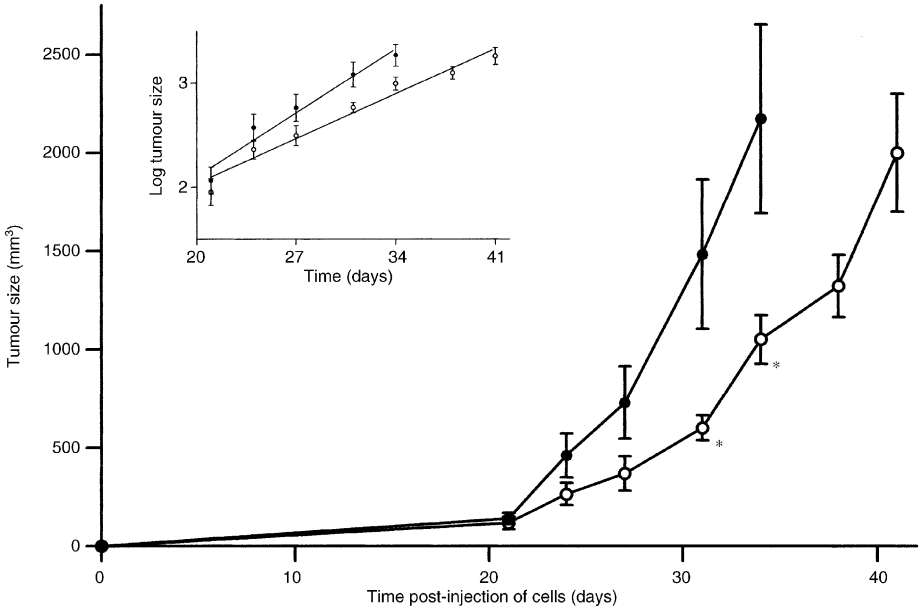

Fig. 2. BFM inhibits the growth of GH3 xenografts. Animals were treated twice weekly with vehicle or BFM (1 mg/kg intraperitoneally (i.p.)) 21 days after subcutaneous (s.c.) injection of GH3 cells when tumours had reached a mean size of at least 100 mm^3. Data shows the mean$\pm$standard error of the mean (SEM) of 7 animals for vehicle-treated (●) and 8 animals for BFM-treated (○), where *$P<0.05$ indicates a significant difference in tumour size for the time point shown (10 and 13 days post-treatment). The inset shows a semi-log plot of tumour size post-treatment demonstrating the different rates of tumour growth.

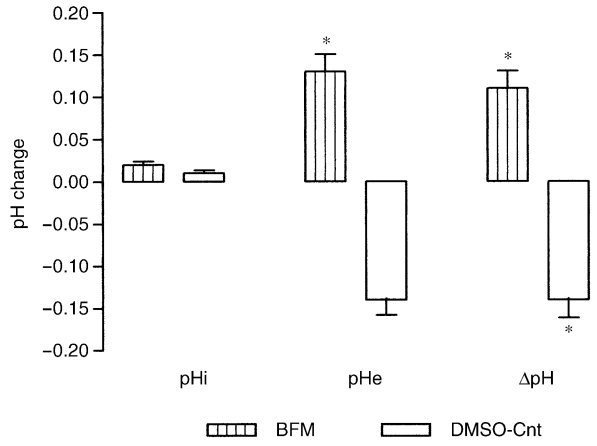

Fig. 3. BFM increases the pHe of GH3 xenografts causing a decrease in the -ΔpH. ^{31}P-MRS was performed on tumours before and 48 h after vehicle or BFM (1 mg/kg i.p.) treatment and pHi and pHe and the consequent pH gradient (ΔpH) determined as described in Methods. Data shows the mean±standard error of the mean (SEM) change in pHi, pHe and ΔpH of 6 animals for BFM-treated and 8 animals for vehicle-treated, where * signifies a significant change over 48 h ($P<0.05$). DMSO-Cnt, control.

NTP/Pi nor PDE/TP ratios were significantly affected by either treatment. In controls, the PME/TP ratio was unaffected, but in 5/6 xenografts BFM induced a small decrease in the PME/TP, although since one tumour showed an increase in the PME/TP ratio and an increase in size over the 48 h, the mean change (−11%) was not significant ($P=0.1$).

Table 2
Morphological status of GH3 xenografts treated by BFM or vehicle

Attributes	Vehicle (7)	BFM (8)
Necrotic	36.8±2.8	38.1±3.0
Vascular	5.9±0.9	4.7±0.4
Cellular	57.3±2.8	57.3±2.8
Apoptotic	1.01±0.12	1.20±0.06
Mitotic	1.97±0.11	1.38±0.12*
Mitotic/apoptotic ratio	2.04±0.16	1.2±0.17*

Data shows mean±SEM of percentages from (*n*) different fixed tumours, where *$P<0.01$ using a Student *t*-test.

3.4. ^{19}F-MRS studies in vivo of effects of BFM on GH3 xenografts

The -ΔpH of tumour cells in culture has been shown to correlate with the intracellular uptake and retention of 5FU (32). ^{19}F-MRS was used to study the uptake and metabolism of 5FU in animals treated 48 hr earlier with BFM or vehicle (control). 5FU appeared rapidly in all tumours and the C_{max} at 10 min and subsequent metabolism to FNuct was not significantly affected by BFM treatment (Fig. 4). However, after the C_{max}, 5FU was eliminated twice as rapidly in the BFM-treated

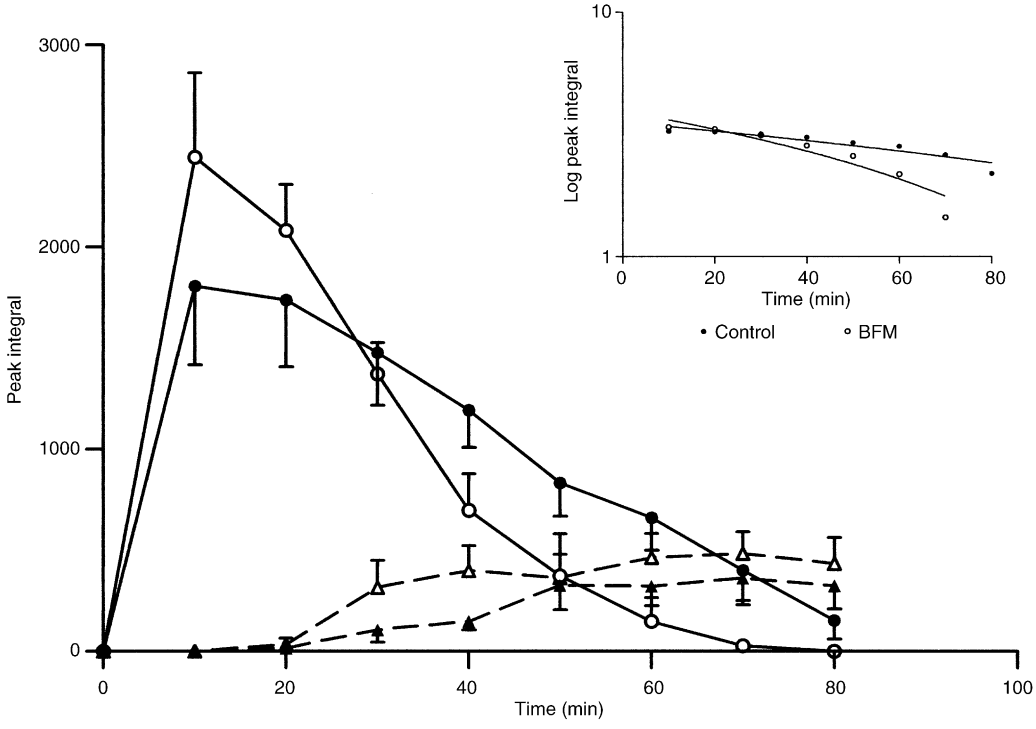

Fig.4. Effect of BFM on 5FU pharmacokinetics in GH3 xenografts. ^{19}F-MRS was performed as described in Methods on tumours immediately following treatment of mice with 130 mg/kg 5-fluorouracil (5FU). 5FU was injected i.p. at 48–50 h after treatment with vehicle or BFM (1 mg/kg i.p.). Data shows the mean±standard error of the mean (SEM) for the peak integrals of 5FU (●) and 5-fluoronucleotides (Fnuct) (▲) for 7 vehicle-treated animals (closed symbols) and 4 BFM-treated animals (open symbols). The inset shows a semi-log plot of the 5FU data for both treatments demonstrating the different rates of elimination of 5FU.

Table 3
An increase in the tumour transmembrane -ΔpH (i.e. a more negative value) correlates with an increased $t_{1/2}$ for elimination of 5FU from tumours grown s.c. in mice

Tumour type	Treatment	Change in ΔpH units	Change in $t_{1/2}$ (min)
Human HT29 colon adenocarcinoma xenograft	IFNα	−0.27	+21.6
Mouse fibrosarcoma RIF-1, (0.8–1.5 g)	Carbogen gas	−0.09	+5.9
Mouse fibrosarcoma RIF-1, (2–3 g)	Carbogen gas	−0.04	+1.2
Rat prolactinoma GH3 xenograft	BFM	+0.11	−8.7

Data for the HT29 and RIF-1 tumours is from earlier publications [27,31]. A plot of the data showed a significant correlation ($r^2=0.994$, $P=0.0033$), with the equation of the line being, $y=80.3-0.8$. IFNα, interferon α.

animals compared with controls, so that the mean $t_{1/2}$ was significantly reduced from 18.1±2.8 to 9.4±1.3 min ($P=0.022$). Thus, a change in the -ΔpH of +0.11 was associated with a change in the $t_{1/2}$ of 5FU of −8.7 min.

The effect of BFM on the 5FU $t_{1/2}$ for elimination from GH3 xenografts is summarised in Table 3 and is compared with other 5FU $t_{1/2}$'s determined previously in different tumour types following various treatments of the mouse host. The data show a pattern consistent with tumour cell data *in vitro* [32], that is, as the -ΔpH increases (i.e. a more negative value), so the retention of 5FU increases i.e. the $t_{1/2}$ increases. Indeed, the limited data set shows a significant correlation ($r^2=0.994$, $P=0.0033$), with the equation of the line being, $y=80.3–0.8$.

3.5. Effect of BFM on total lactate levels of GH3 tumours

In tumours grown in either mice or rats, BFM treatment after 48 hr caused a significant mean decrease in total lactate of approximately 28% compared with DMSO-treated controls. In rats, lactate was reduced from 5.4±0.4 ($n=7$) to 4.0±0.2 mM ($n=8$), $P=0.04$, and in mice from 6.4±0.7 [7] to 4.6±0.4 mM, [7] $P=0.046$.

4. Discussion

We have shown that the macrolide antibiotic BFM is a potent inhibitor of cell proliferation *in vitro* with IC_{50}'s in the low nM range for a number of animal and human cell lines. In rat pituitary GH3 tumour cells, BFM induced an increase in the proportion of cells in S and G2M phases after 16–24 h incubation and there was no evidence of apoptosis. BFM also inhibited the growth of GH3 mouse xenografts via a reduction of mitosis, but again did not affect apoptosis. In GH3 xenografts, BFM caused an increase in pHe without affecting pHi and consequently reduced the -ΔpH. In both rat and mouse GH3 tumours, BFM significantly reduced the total lactate. Finally, consistent with previous reports (see below), the reduced -ΔpH was associated with a significant decrease in the $t_{1/2}$ for elimination of 5FU from the tumour. The precise mechanism of these effects is unknown, but we propose that BFM inhibited the export of H^+ from the intracellular compartment to the extracellular compartment by inhibition of vacuolar H^+-ATPases (V-ATPases) functionally expressed in the plasma membrane.

Over 70 years ago, it was shown that tumour cells have high levels of glycolysis even in the presence of oxygen [33] and the phenomenon is observed in both adherent (solid) and suspension (haematological) cells. Gatenby [4] proposed that in solid tumours glycolysis may confer a growth advantage over surrounding cells by causing an increased H^+ and lactate production leading to a low pHe following export from the intracellular compartment. The low pHe would inhibit the metabolism of normal cells and promote release of lysosomal enzymes leading to a breakdown of the extracellular matrix. Indeed, in cell culture it has been shown that a low pHe increases the production of angiogenic factors (8) and increases the invasive behaviour of human tumour cells [7]. A number of different carriers are involved in regulating pHi of mammalian cells including the monocarboxylate lactate carrier, Na^+/H^+ antiport, the $HC0_3^-/Cl^-$ exchanger as well as perhaps ATP-dependent processes such as the K^+/Na^+ exchanger and vacuolar H^+-ATPases [1]. The V-ATPases are normally considered to be involved in acidifying lysosomes and endosomes, but the enzymes may also exchange dynamically with the cell plasma membrane [18], and play a role in protein degradation [34], the transferrin cycle [14], multi-drug resistance [17,18] and plasma membrane H^+ transport [6,11]. High levels of V-ATPase mRNA have been detected in human pancreatic tumour cells and this was associated with increased metastasis *in vivo* [16]. Perhaps because the V-ATPase is not statically resident in the plasma membrane, it has not yet been detected by antibodies in the plasma membrane of tumour cells [18], but a range of data *in vitro* suggests they are functionally expressed [6,11,17]. We hypothesised that a potent inhibitor of V-ATPases might impact on H^+ transport by tumour

cells resulting in an increase in tumour pHe and thus reduce tumour growth. BFM was chosen since it is a potent and highly specific inhibitor of V-ATPases prepared from renal chromaffin granules [10] or osteoclast plasma membranes [35].

^{31}P-MRS showed that BFM did not affect the pHi, but caused an increase in pHe of 0.13 units resulting in a decrease in the -ΔpH, while in controls there was an equivalent decrease in pHe and the -ΔpH was consequently increased. The large decrease in control pHe was puzzling, but suggested that the vehicle (DMSO) was not entirely inert and may therefore have altered host physiology. This also implied that the vehicle masked an even greater effect of BFM on the pH. Nevertheless, taken together the results suggest that 1 mg/kg i.p. of BFM had a significant biological impact on the tumour extracellular [H^+]. Other parameters measurable by ^{31}P-MRS were not significantly affected, although 5/6 xenografts examined in BFM-treated animals showed a small decrease in PME/TP, which would be consistent with a decrease in cell proliferation [36]. The effect of higher doses of BFM on tumour pH were not tested due to rapid lethality.

The change in the pHe of GH3 xenografts caused by BFM was associated with a number of other marked alterations in GH3 tumour biology including growth and mitosis, lactate formation and 5FU pharmacokinetics. Tumour growth was significantly inhibited causing a growth delay of approximately 60%. Although this was a relatively modest effect, there was a significant reduction in the number of mitoses, which fitted with the increase in G2M elicited by BFM on isolated GH3 cells. However, neither the histology nor the flow-cytometry showed any evidence for an increase in apoptosis induced by BFM. Thus, in this particular model, BFM appeared to inhibit cell proliferation by arresting DNA synthesis and mitosis without inducing apoptosis. In contrast, others have reported that similar concentrations of BFM induced apoptosis in cell cultures of mouse lymphoma cells [37] and neural (PC12) cells [15] and in human pancreatic xenografts *in vivo* [16]. There is increasing evidence that cells can die in response to chemotherapy by means other than classic apoptosis [38] and this would be dependent on the particular signal transduction pathways that are relevant in that cell. For example, rapamycin can induce apoptosis in human cell lines that have a mutated p53 because there is no G1 arrest and the cells progress into the cycle leading to apoptosis, but in wt-p53 cells, the G1 arrest leads to a cytostatic effect of rapamycin [39]. The p53 status of GH3 cells is not clear, but they do express high levels of bcl-2 protein, a negative regulator of apoptosis [40]. Furthermore, since BFM is not a DNA-damaging agent it is perhaps less surprising we do not see classical apoptosis, while an interaction with the mitochondria is unknown, but unlikely given the specificity of BFM for V-H^+-ATPases, thus making pro-apoptotic signals improbable. We propose that BFM has a cytostatic type effect on the GH3 tumour cells through a G2M block perhaps as a consequence of reduced glycolysis (see below).

In GH3 tumours grown in both rats and mice, BFM significantly reduced the total level of lactate (intra plus extracellular) suggesting a decrease in the rate of glycolysis. The end product of glycolysis is lactate plus H^+, and these products need to be efficiently exported from the cell so as to maintain pHi and other vital processes. The cell has many different systems for maintaining pHi (see above and Stubbs et al, 2000 [1]), and inhibition of one of these, e.g. H^+ export by V-ATPases, would probably cause upregulation by one or more of the other systems. Nevertheless, we observed a significant increase in pHe, implying that reduced H^+ transport occurred, but since pHi was unaffected, the response of the cell to the increased intracellular [H^+] may have been to reduce glycolysis by simple negative feedback from the accumulation of the products. Thus, in GH3 tumours we hypothesise that BFM may inhibit tumour growth by two separate, but related, mechanisms; the reduction in glycolysis would reduce the energy available for cell proliferation and the increased pHe would reduce angiogenesis and consequent spread of tumour cells at the expense of normal cells. We suggest that an increase in tumour pHe and perhaps specifically inhibition of V-ATPase function might provide a novel molecular target for inhibiting tumour growth.

Finally, the reduced -ΔpH induced by BFM was associated with a more rapid elimination of 5-FU from the tumour i.e. a shorter $t_{1/2}$. While it is possible of course that BFM treatment impacts on other aspects of cell and tumour physiology and this may indirectly alter the tumour ΔpH and drug elimination too, we have made related observations in other solid tumour models *in vivo* in which an *increase* in the -ΔpH was associated with an increase in 5FU retention by solid tumours, i.e. a longer $t_{1/2}$ for drug elimination [27,31]. Thus, these experiments *in vivo* on three different tumour types are entirely consistent with the data obtained on two different types of isolated tumour cells which showed that 5FU uptake was correlated to the -ΔpH [32], i.e. the more negative the ΔpH, the greater the tumour retention of 5FU. Indeed, the collated data *in vivo* shown here demonstrate a strong correlation between these two parameters, and the equation of the line predicts that an increase in the -ΔpH of 0.1 units would increase the $t_{1/2}$ by 7.2 min. This has important implications for the clinic, since Wolf and colleagues [41] have shown a strong association between the 5FU tumour $t_{1/2}$ and patient response to 5FU for a large range of solid tumours. Our data suggests that variations in pH of solid tumours in the clinic may play a role in drug trapping, implying treatments that increase the tumour

-ΔpH would increase patient response to 5FU treatment. Similarly, any drug treatments that lead to a reduction of the -ΔpH, whether directly or indirectly, may decrease retention of 5FU in the tumour and this is an important consideration in the clinic where 5FU is used in combination with a number of different drugs. Finally, our observations here, earlier [27,31] and those of others [42,43] have demonstrated that the tumour ΔpH *can* be manipulated *in vivo* and thus retention and consequent cytotoxicity of some drugs, including 5FU, can be strongly influenced by modulation of the tumour microenvironment.

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