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ORIGINAL ARTICLE

A simple ex vivo bioassay for 5-FU transport into healthy buccal mucosal cells

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Abstract

Purpose Fluorouracil (5-FU), a chemotherapeutic agent widely used in the treatment of numerous common malignancies, causes oral mucositis in a proportion of patients. The contribution of drug transport processes to the development of this toxicity is currently unknown. This work aimed to establish and optimise a simple phenotyping assay for 5-FU uptake into primary buccal mucosal cells (BMC).

Methods The uptake kinetics of radiolabelled 5-FU were determined in pooled BMC freshly collected from healthy volunteers. The inter- and intra-individual variability in 5-FU uptake was then assessed across a cohort that included both healthy volunteers and cancer patients.

Results 5-FU uptake into pooled primary BMC was both time and concentration dependent. An Eadie–Hofstee analysis suggested two components; a high-affinity ($K_M = 3.3$ µM) low-capacity ($V_{MAX} = 57.8$ pmol min^{-1} 10^5 viable cells^{-1}) transporter, and a high-capacity ($V_{MAX} = 1230$ pmol min^{-1} 10^5 viable cells^{-1}) low-affinity ($K_M = 3932$ µM) transporter. There was 180-fold variation in the rate of 5-FU uptake into BMC (0.10–17.86 pmol min^{-1} 10^5 viable cells^{-1}) across the 34 subjects (healthy participants $N = 24$, cancer patients $N = 10$). Notably, retesting of a subset of these participants ($N = 16$) multiple times over a period of up to 140 days demonstrated poor stability of the uptake phenotype within individuals.

Conclusion The uptake of 5-FU into healthy oral mucosal cells is a highly variable process facilitated by membrane transporters at pharmacologically relevant concentrations. This bioassay is simple, minimally invasive, and suitable for phenotypic analysis of drug transport in healthy primary cells.

Keywords Cancer pharmacology · 5-Fluorouracil · Mucosal cells · Drug transport · Drug toxicity · Phenotype

Introduction

Fluorouracil (5-FU), a rationally designed fluorinated analogue of the endogenous pyrimidine nucleobases uracil and thymine, is an effective chemotherapeutic agent used widely in the treatment of gastrointestinal and metastatic breast cancers. However, use of 5-FU is often associated with significant normal tissue toxicities. Ulceration and inflammation of the mucosa (mucositis) is common and of substantial clinical concern. 5-FU is particularly mucotoxic, with evidence of mucositis in up to 97% of patients following treatment with 5-FU containing drugs [1]. Reports of mucositis in patients are most frequently specific to the oral cavity since this site can be readily evaluated for ulceration, however it can affect any region of the alimentary tract and may result in additional symptoms including nausea, vomiting, abdominal pain, and diarrhoea. The diagnosis of mucositis in the gastrointestinal tract commonly relies on these non-specific indicators of gastrointestinal injury, as this site is more difficult to directly visualise than the oral cavity. Gastrointestinal symptoms may be confounded by other clinical factors [2], and not all patients presenting with diarrhoea have concomitant oral mucositis [3, 4].

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Inherited deficiencies in dihydropyrimidine dehydrogenase (DPD) activity, the rate limiting enzyme for pyrimidine nucleobase catabolism, are associated with the side effects of 5-FU in some individuals. However, known genetic polymorphisms in the *DPYD* gene, which encodes DPD, are found in only a quarter of patients presenting with severe 5-FU toxicity [5–11]. The factors that contribute to the development of severe gastrointestinal toxicity in those patients who have not inherited DPD deficiency are at present poorly understood. Although the contribution of other metabolic enzymes to 5-FU toxicity have been investigated (most notably methylene tetrahydrofolate reductase and thymidylate synthase), the potential role of 5-FU cellular transport processes in fluoropyrimidine mucosal toxicity has been almost entirely overlooked. 5-FU concentrations have been shown to be at least tenfold higher in both normal (gastrointestinal mucosa, liver) and tumour tissues (primary gastrointestinal and liver metastases) than in plasma following dosing by intravenous bolus, suggesting concentrative 5-FU transport occurs in these cells [12].

The membrane-bound solute carrier (SLC) transporter superfamily are responsible for the cellular uptake of a wide array of nutrient and xenobiotic substrates, including inorganic ions and both charged and uncharged organic molecules. Despite their physiological importance, their role in oncology is often overlooked in favour of efflux transporters and metabolic enzymes. Pyrimidine nucleobases, including 5-FU, are water-soluble compounds that are unlikely to cross cell membranes without some form of facilitated transport. Little research has been previously undertaken to study 5-FU transport in normal tissues, perhaps in part due to the ethical and practical difficulties associated with obtaining live primary cells from many tissues in healthy donors. Additionally, in vitro transport studies commonly rely on the use of transformed cell lines and/or artificial expression systems. As such, the identity of the human transporter(s) specific for carrier-mediated uptake of 5-FU in normal mucosal tissues has not been confirmed. Buccal mucosal cells, however, are an easily accessible tissue susceptible to 5-FU toxicity that can be collected using minimally invasive techniques [13]. We present here an assay to measure 5-FU transport into ex vivo buccal mucosal cells for potential use both to investigate the specific mechanisms that regulate and facilitate 5-FU transport in normal human cells, and as a minimally invasive transport phenotyping test in both healthy volunteers and cancer patients.

Materials and methods

Approvals were obtained from the New Zealand Northern A Health and Disability Ethics Committee for buccal mucosal cell sampling of cancer patients (14/NTA/186) and healthy volunteers (15/NTA/14, 17/NTA/160). The cancer patients were recruited as an optional sub-study of a larger observational clinical trial (Australian New Zealand Clinical Trials Registry: ACTRN12615000586516). All study participants were required to be ≥ 18 years old at the time of recruitment and able to provide written informed consent. Cancer patients were also required to have histologically confirmed gastrointestinal or metastatic breast cancer, and to be scheduled to receive (or have received) 5-FU or capecitabine as single agent therapy. Exclusion criteria included current medication use or illness for the healthy volunteers, concurrent radiation therapy for the cancer patients, and current pregnancy or breastfeeding (all participants).

Following informed consent, 24 healthy participants and 10 cancer patients were recruited to the study (Table 1). The 21 (61.8%) female and 13 (38.2%) male participants had a median age of 48 years (range 20–74 years). While there was no significant difference in gender ratio, the healthy volunteers (median 29 years, range 20–74 years) were significantly ($p = 0.0006$) younger than the cancer patients (median 61 years, range 43–74 years). The majority of participants self-identified as European (66.7%), with Asian (23.5%) and NZ Maori (5.9%) descent also reported. The cancer patients recruited were in receipt of capecitabine monotherapy for metastatic breast and/or gastrointestinal cancer diagnoses, however all cell samples were collected either prior to commencement of chemotherapy, following a dose holiday, or after completion of treatment to minimise confounding of the study results due to systemic fluoropyrimidine exposure.

Buccal mucosal cells (BMC) were collected by cytobrush [13], and immediately transferred into 5 mL preheated (37 °C) uptake buffer containing 140 mM sodium chloride (NaCl), 5 mM potassium chloride (KCl), 0.4 mM potassium dihydrogen phosphate (KH_2PO_4), 0.8 mM magnesium sulphate ($MgSO_4$), 1.0 mM calcium chloride ($CaCl_2$), 25 mM glucose, and 10 mM HEPES, pH 7.4 [14]. The drug uptake assay was undertaken directly following cell isolation. Cell number and viability was assessed by trypan blue exclusion on a Neubauer haemocytometer, before the cell suspensions were aliquoted into 2 mL microcentrifuge tubes. Where possible, the total BMC cell count was maintained at $\geq 1.5 \times 10^5$ cells per aliquot in order to sustain adequate cell pelleting throughout the transport assay. When the number of cells collected was sufficient, technical replicates were utilised to enhance the reliability of the data. Any data obtained from < 3 technical replicates is noted.

Cell pellets were prepared by centrifugation (1600*g*, 5 min) and the uptake buffer discarded. Cells were then resuspended in 100 µL uptake buffer (37 °C) containing [^{14}C] 5-FU (specific activity; 10,360 Bq/mL, > 99% purity, American Radiolabeled Chemicals, MO), with a matched ice-cold incubation undertaken simultaneously for all participants. Following incubation, the cell suspension was immediately

Table 1 Demographics of the 34 participants recruited to the study

	Healthy volunteers ($N=24$)	Cancer patients ($N=10$)	p value	Total ($N=34$)
Diagnosis				
Gastrointestinal cancer	0	4	–	4
Metastatic breast cancer	0	5		5
Metastatic breast and gastrointestinal cancer	0	1		1
Sex (self-reported)				
Female	14	7	0.7041	21
Male	10	3		13
Age [median (range)][a]	29 (20–74) years	61 (43–74) years	0.0006	48 (20–74) years
Ethnicity (self-reported)[a,b]				
European	17	5	0.1229	22
Other	5	5		10

[a]Age and ethnicity data were not recorded for three healthy volunteers

[b]Individuals were permitted to report multiple ethnicities

placed on ice and diluted with 900 µL of ice-cold buffer. The cells were then pelleted (1600*g*, 5 min) and the buffer discarded. The cell pellet was washed twice to remove any remaining extracellular [^{14}C] 5-FU as follows: cells were resuspended in 500 µL of ice-cold buffer, then centrifuged (1600*g*, 5 min) to pellet the cells and facilitate removal of the buffer. Cell pellets were then lysed in 1 mL of 1% *w*/*v* SDS in 0.1 M sodium hydroxide and the lysate transferred into 6.5 mL scintillation vials (Sigma–Aldrich, MT, USA). Emulsifier Safe scintillation fluid (5 mL, PerkinElmer, MA, USA) was added to the lysate and the tubes gently inverted to mix the components. The sample was analysed on a Tri-Carb® 4910TR liquid scintillation analyser (PerkinElmer, MA, USA), with the data recorded as [^{14}C] disintegrations per minute (DPM). Uptake of radiolabelled drug was calculated from these data and the amount was normalised to the number of viable cells in the pellet.

Using this assay, the time course (0, 2, 4, 6, 8, 10, 15, 20, 25, 30, 40, 50, and 60 min incubation times) and substrate concentration (0, 5, 10, 50, 100, 500, 1000, 5000, 10,000 µM) kinetics of 5-FU uptake were determined in BMC pooled from 6 healthy individuals. Three independent repeat experiments were performed for each assay. On the basis of these results, assessment of individuals' 5-FU uptake into BMC was undertaken using a drug concentration of 5 µM and an incubation time of 5 min.

Statistical and regression analyses were performed using GraphPad Prism software (Version 8, GraphPad Software Inc.). Variability in population data across the study cohort is reported as the mean ± standard deviation (SD), while the uncertainty of measurements is described as the mean ± standard error of the mean (SEM) of either *n* technical replicates or *N* independent repeat experiments, as appropriate. Time course data was modelled with either a one-phase association curve or a linear regression, as appropriate. Modelling of the Eadie–Hofstee plot components was undertaken by linear regression, before Michaelis–Menten saturation curves were fitted to the concentration data. The maximum rate of uptake (V_{MAX}) and Michaelis–Menten constant (K_M) were calculated using these models.

Normality testing of the study population data was undertaken using the D'Agostino–Pearson omnibus K2 normality test ($\alpha = 0.05$). Non-parametric population data are reported as the median (interquartile range, IQR), and comparisons between groups undertaken using the Mann–Whitney test. Non-Gaussian data was $\log_{10}$ transformed in order to undertake parametric statistical analyses, where that transformed data passed normality testing (i.e. the data was lognormal). In those cases, differences between two groups were evaluated using either unpaired *t* tests with Welch's correction or paired *t* tests, as appropriate, and between three or more groups using 1-way ANOVA with Tukey's multiple comparisons test. Temporal trends in the log transformed rates of uptake were estimated by least squares regression analysis, with the adequacy of this model assessed by replicates testing. $p < 0.05$ was considered statistically significant.

Results

Viable mucosal cells were observed in all buccal samples collected, and ranged from 12.3 to 71.4% of the total cell count across the participants (median = 25.0%, IQR = 17.0–33.5%). Although there was no significant difference in the viable cell counts between the two groups, the median total cell number collected was significantly higher

($p = 0.0071$) in the healthy participants than in the cancer patients (11.5×10^5 cells vs 8.1×10^5 cells).

Initial experiments were undertaken to investigate the effects of time and concentration on the kinetics of 5-FU uptake into pooled primary BMC ex vivo. [^{14}C] 5-FU was detectable in BMC cell lysates at all time-points and drug concentrations studied. The precision of the assay was acceptable ($C_V = 18.0\%$ across $n = 6$ technical replicates). Uptake into BMC was time-dependent; accumulation of the drug plateaued over a 60 min time course (Fig. 1a) and was linear for incubation times ≤ 10 min (Fig. 1b). The maximum concentration of 5-FU in cell lysate was calculated to be 96.7 ± 15.8 pmol 10^5 viable cells^{-1} across the three independent experiments undertaken. An incubation time within the linear portion of the curve (5 min) was subsequently selected to assess the effect of substrate concentration on uptake into pooled BMC (Fig. 1c). While the rate of 5-FU uptake was consistent with Michaelis–Menten kinetics and tended toward saturation with increasing concentration (0–10,000 µM 5-FU) an Eadie–Hofstee plot of this data resolved into two linear components (Fig. 1d), indicating that the uptake of 5-FU into BMC may involve multiple transport processes. One component (Fig. 1e) exhibited high affinity for 5-FU ($K_M = 3.3$ µM) but low transport capacity ($V_{MAX} = 57.8$ pmol min^{-1} 10^5 viable cells^{-1}), while the other (Fig. 1f) had high capacity for uptake of the drug ($V_{MAX} = 1230$ pmol min^{-1} 10^5 viable cells^{-1}) but approximately 1000-fold lower 5-FU affinity ($K_M = 3932$ µM).

In order to validate the utility of the assay to for analysis of individuals' 5-FU uptake into BMC, both inter- and intra-individual variability were assessed. For this work we selected an incubation time (5 min) and pharmacologically relevant substrate concentration (5 µM, [15–18]) that fell within the linear portion of the relevant kinetics curve (time course and low concentration Michaelis–Menten curve, respectively), to ensure that the results were not confounded by saturation of the system. Uptake of [^{14}C] 5-FU was successfully assayed in all study participants, however BMC collection yielded insufficient numbers of cells to undertake triplicate analysis for two healthy individuals (analysed in duplicate) and two cancer patients (single replicate each). The frequency distribution of the rate of uptake across the 34 participants was positively skewed and differed significantly from a normal distribution (Fig. 2a; $p = 0.0176$). Importantly, the rate of uptake into BMC from different donors appeared to vary substantially, ranging from 0.10 to 15.27 pmol min^{-1} 10^5 viable cells^{-1} in healthy individuals and 0.17–17.86 pmol min^{-1} 10^5 viable cells^{-1} in cancer patients (Fig. 2b).

Following logarithmic transformation the data did not differ significantly from a Gaussian distribution ($p = 0.3096$); parametric statistical analyses were therefore undertaken on the $\log_{10}$ transformed data. There was no significant difference in the mean $\log_{10}$ rate of 5-FU uptake between healthy individuals and cancer patients ($p = 0.9921$). These data remained non-significant when cancer patients were stratified by diagnosis ($p = 0.2940$). There was also no correlation between age and uptake (Pearson's $r = 0.11$, $p = 0.5559$), and no difference between males and females ($p = 0.9649$) or across ethnicities ($p = 0.8562$). Uptake of 5-FU was temperature dependent; the $\log_{10}$ rate of uptake across the population was significantly higher at 37 °C than in matched incubations performed on ice ($p < 0.0001$).

Repeated phenotype testing was undertaken in a subset of the healthy participants ($N = 16$) on at least 2 and up to 10 additional occasions to investigate inter-occasion changes in the transport of 5-FU into BMC. Summary box plots of the median, IQR, and range of 5-FU uptake rates for each participant are presented in Fig. 3a. Assessment of the intra-individual stability of the uptake phenotype by linear regression of the $\log_{10}$ transformed data showed no clear overall trend: rates of uptake decreased over time in 2 (12.5%) volunteers, increased in 4 (25.0%) volunteers, and had no significant trend in 10 (62.5%) volunteers (Table 2). It should be noted that the r^2 values were > 0.5 for only three of these trend lines and that linear regression was an inadequate model of the data in the majority (81.3%) of cases. The intra-individual variability underlying this was pronounced, with a mean inter-occasion C_V of $139.1\% \pm 175.7\%$ across the group. Furthermore, the rate of uptake at the first sample (day 0) was poorly predictive for subsequent testing occasions, approximating the median for only one individual (#0014) and falling outside the IQR entirely for 6 (37.5%) participants (Fig. 3a, grey circles). Substantial changes (≥ 50% increase or decrease from day 0) were observed on multiple occasions in all individuals. These data suggest a spot test would have poor predictive power to determine an individual's average rate of 5-FU uptake into BMC over time. In the most extreme example (#0011), decreases of up to 94% (day 21; 0.04 ± 0.01 pmol 10^5 viable cells^{-1}) and increases of up to 44-fold (day 143; 27.26 ± 3.74 pmol 10^5 viable cells^{-1}) were observed in comparison to the day 0 rate of uptake (0.62 ± 0.05 pmol 10^5 viable cells^{-1}). This individual was sampled concurrently with three others (#0002, #0012, #0015) over a period of 20 weeks (Fig. 3b), with the uptake assay performed simultaneously on each occasion by a single investigator. This group also included, by chance, the two individuals with both the highest (#0002) and lowest (#0015) median uptake rates (18.6 vs 0.4 pmol 10^5 viable cells^{-1}), and the widest (#0002) and narrowest (#0015) ranges (2.5–54.1 vs. 0.1–2.0 pmol 10^5 viable cells^{-1}). There were no associations between either the inter-occasion or the inter-individual variability and the frequency of sampling or the day of analysis.

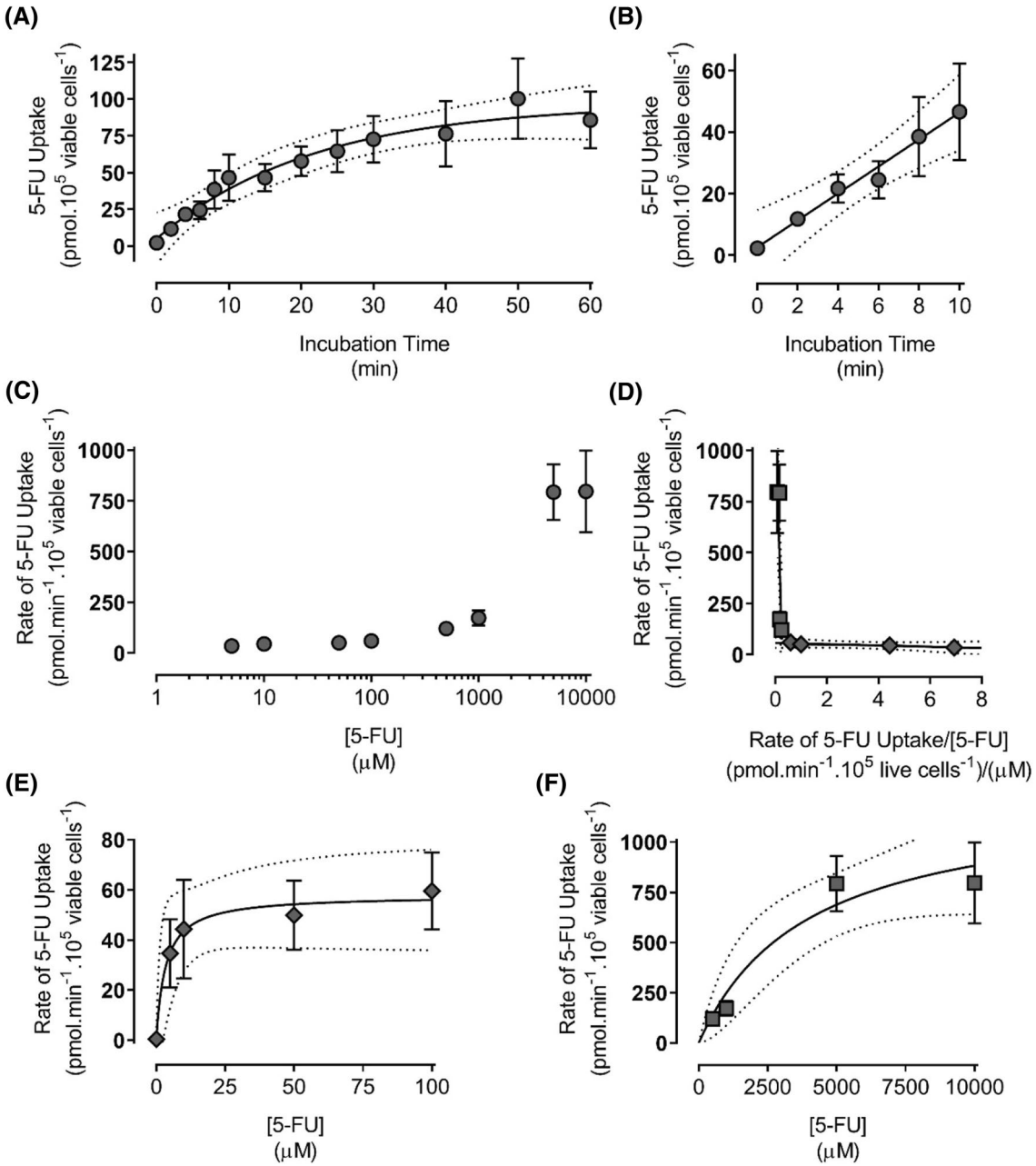

Fig. 1 **a** Time course of [^{14}C] 5-FU uptake into primary buccal mucosal cells pooled from 6 healthy participants (ID: 0001, 0002, 0003, 0006, 0011, 0012) over 60 min. Data are fitted with a one-phase association curve ($y = 5.014 + (96.67 - 5.014)\left(1 - e^{-0.04596x}\right)$, $r^2 = 0.6349$). **b** Initial rate of uptake was linear up to 10 min incubation time ($y = 4.380x + 2.514$, $r^2 = 0.5925$). **c** Effect of substrate concentration on the rate of [^{14}C] 5-FU uptake into pooled buccal mucosal cells from 6 healthy participants (0001, 0002, 0003, 0004, 0005, 0012). **d** Eadie–Hofstee plot of the variable substrate concentration data, with linear regressions fitted across two apparent components (diamonds: $y = -3.257x + 57.69$, $r^2 = 0.1238$; squares: $y = -4631x + 1231$, $r^2 = 0.5010$). **e** Re-analysis of the rate of 5-FU uptake at low substrate concentrations (≤ 100 μM, diamonds), fitted with a Michaelis–Menten curve ($y = \frac{57.83x}{3,306+x}$, $r^2 = 0.5000$). **f** Re-analysis of the rate of 5-FU uptake at high substrate concentrations (≥ 500 μM, squares), fitted with a Michaelis–Menten curve ($y = \frac{1230x}{3932+x}$, $r^2 = 0.8009$). All graphs: Data are the mean ± SEM of $N = 3$ independent repeat experiments, with $n = 3$–4 technical repeats undertaken per experiment, as cell number permitted. Regressions (solid lines) were calculated taking into account the N and scatter among replicates, with 95% confidence intervals indicated by dotted lines. Where not visible, error bars are smaller than the symbol

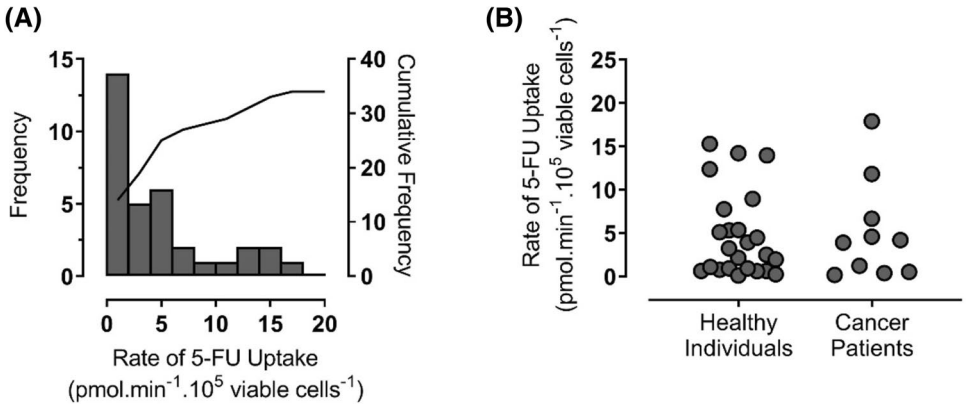

Fig. 2 **a** The frequency distribution of the rate of [^{14}C] 5-FU uptake into BMC across the total study population ($n=34$). The cumulative frequency of the data is indicated by the black curve. The median rate of uptake across all participants was 3.56 pmol min^{-1} 10^5 viable cells^{-1} (IQR = 0.74–6.93 pmol min^{-1} 10^5 viable cells^{-1}). **b** The range of rates of [^{14}C] 5-FU uptake into BMC in healthy individuals ($n=24$) and cancer patients ($n=10$). Data points represent the mean of 3 technical replicates for each individual, with the following exceptions; due to low cell number at sample collection volunteers 0024 and 0026 were analysed in duplicate and only a single technical replicate each was achieved for patients 4031 and 4036

Discussion

Oral mucositis is prevalent and sometimes treatment limiting in a substantial proportion of patients receiving 5-FU based chemotherapy. The expression and localisation of cell membrane uptake transporters can be highly variable, which could theoretically contribute to the inter- and intra-individual variability observed in the clinical incidence of 5-FU induced mucositis. We have established a simple bioassay to measure 5-FU uptake into freshly collected primary human cells in suspension, in an easily accessible cell type from a tissue site relevant to this toxicity (buccal mucosal cells). This uptake is carrier-mediated, saturable, and appears to consist of both high affinity–low capacity and low affinity–high capacity components. The high-affinity component ($K_M = 3.3$ µM) may be relevant at plasma 5-FU concentrations observed following continuous infusion of the drug (3-9 µM) or after capecitabine p.o. (approx. 2 µM) [15–18]. In contrast, the low-affinity component ($K_M = 3.9$ mM) may be pertinent in individuals in receipt of intravenous bolus dosing, when peak plasma concentrations of up to 1.2 mM 5-FU occur [19, 20]. Additionally, the uptake of 5-FU into buccal cells was minimal when cells were incubated with the drug on ice. Cryotherapy (ice chips held in the mouth before (5–10 min), during, and/or after (10–35 min) bolus intravenous 5-FU) can be used to effectively minimise oral mucositis in patients [21–25]. It has been assumed that ice-induced vasoconstriction minimises exposure of the cells to systemic 5-FU, however it may be that this approach also decreases the activity of the transporters(s) responsible for cellular uptake of 5-FU.

Several SLC transporters have been identified that are capable of 5-FU uptake in in vitro systems and/or animal models. The organic anion transporter SLC22A7 (OAT2) can facilitate transport of 5-FU with high affinity ($K_M = 54$ nM) in a *Xenopus* oocyte expression system [26], and high tumour OAT2 expression correlates with a good response to 5-FU based chemotherapy (uracil/ftorafur ± leucovorin, 5-FU/leucovorin/oxaliplatin) [27, 28]. OAT2 expression appears to be largely confined to the kidneys and liver in humans, and includes differentially localised splice variant proteins with distinct substrate specificities [29]. Its stoichiometry is unknown, and no crystal structure has been published to date. The equilibrative nucleoside transporters SLC29A1 (ENT1) and SLC29A2 (ENT2) are also capable of facilitated uptake of pyrimidine nucleobases, including 5-FU ($K_M = 2.3$ mM and $K_M = 2.6$ mM, respectively), in *Xenopus* oocyte models [30]. High *SLC29A1* mRNA expression in human colorectal tumour biopsies has also been correlated with 5-FU effectiveness [31], and a role for rat Slc29a2 has been suggested for the transport of 5-FU in both rat trophoblasts and transfected *Xenopus* oocytes [32]. Both are ubiquitously expressed across tissue types in humans [33–35]. An additional sodium-dependent concentrative transporter specific for pyrimidine nucleobases (Slc23a4) has been identified in the small intestine of rats [14]. However, its putative human ortholog (*SLC23A4P*) is a pseudogene lacking several exons coding transmembrane domains [14]. Whether one or more of these candidates, or another as yet unidentified transporter, mediate 5-FU uptake into BMC is currently unknown. The active and/or facilitated transport of drugs in BMC is largely understudied, perhaps because a substantial proportion of saliva–blood transport is thought to occur via the paracellular pathway [36].

Notably, we found 180-fold between-subject variability in 5-FU uptake into BMC. This inter-individual variation

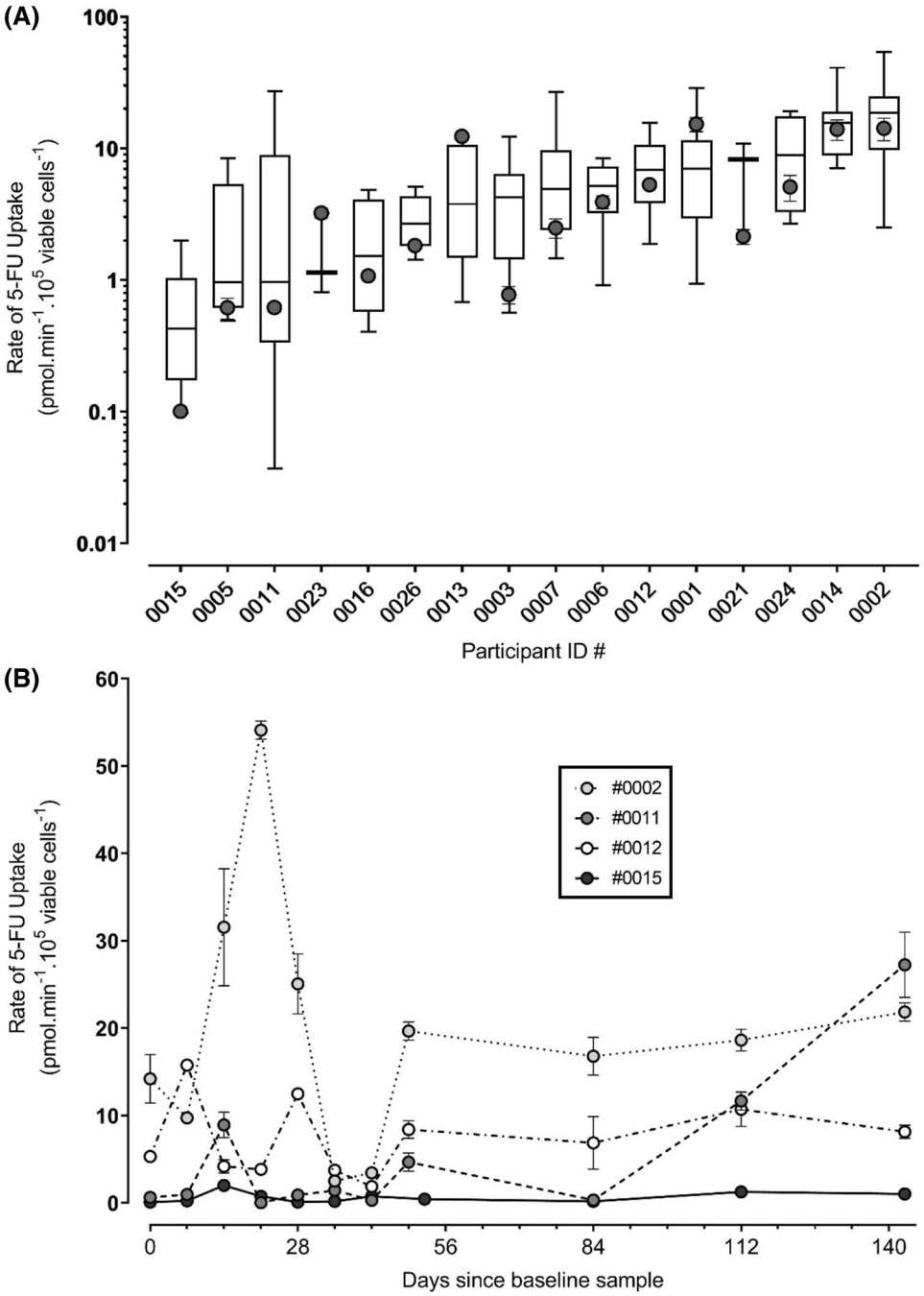

Fig. 3 **a** Variability in the rate of 5-FU uptake into buccal mucosal cells within $n = 16$ participants over multiple test occasions. Data are presented as box and whisker plots of the median, IQR, and range for each individual, with their baseline (day 0) rate of uptake indicated by the grey circle (mean ± SEM of n technical replicates). The *y* axis is plotted on a $\log_{10}$ axis for clarity. **b** A representative example of the inter-occasion variability observed in four of these participants (#0002, #0011, #0012, #0015), who were assayed concurrently 11 times over approximately 20 weeks. Data points represent the mean ± SEM of 2–4 technical replicates for each test occasion, as permitted by the number of cells collected at each test occasion. Where not visible, error bars are smaller than the symbol

was not normally distributed, a pattern often suggestive of genetic polymorphism. However, a simple genetic (SNP-based) cause for this difference is unlikely, since repeated phenotype testing highlighted the substantial inter-occasion (intra-individual) variation in cellular transport of 5-FU. These data suggest that this process may instead be influenced by environmental or regulatory factors. The impact of changes in uptake on this scale on the toxicity of 5-FU to BMC are currently unknown, however it is plausible that substantial increases in uptake of the drug may outpace its catabolic metabolism by DPD in this cell type.

Assessment of DPD activity in peripheral blood mononuclear cell (PBMC) lysate correlates with the rate of hepatic 5-FU catabolism in patients, the major route of clearance for this drug [37]. Importantly, the activity of the DPD enzyme is reported to be substantially lower in BMC lysate (healthy volunteers: 1.7 ± 1.0 nmol mg^{-1} h^{-1}, $n = 11$; cancer patients: 1.9 ± 1.5 nmol.mg^{-1} h^{-1}, $n = 24$ [6]) compared with PBMC lysate, not only from patients with normal DPD activity (9.6 ± 2.6 nmol mg^{-1} h^{-1}, $n = 44$ [6]; 9.9 ± 3.5 nmol mg^{-1} h^{-1}, $n = 28$ [38]), but importantly from patients with known *DPYD* null function variants

Table 2 Inter-occasion variability in the log-transformed rate of 5-FU uptake into buccal mucosal cells across multiple testing occasions

Log rate of 5-FU uptake $\log_{10}$ (pmol min^{-1} 10^5 viable $cells^{-1}$)							
ID #	Inter-test variation		Number of test occasions	Linear regression	r^2	Does the slope deviate from zero?	Is the model adequate?
	Mean ± SD	C_V (%)					
0001	0.75 ± 0.44	58.0	9	$y = -0.004184x + 1.084$	0.1663	Yes ($p = 0.0347$)	No ($p < 0.0001$)
0002	1.16 ± 0.39	34.0	11	$y = 0.0008193x + 1.121$	0.0087	No ($p = 0.6064$)	No ($p < 0.0001$)
0003	0.49 ± 0.45	91.6	10	$y = 0.001026x + 0.3816$	0.0148	No ($p = 0.5218$)	No ($p < 0.0001$)
0005	0.19 ± 0.48	259.1	7[a,b]	$y = 0.002730x + 0.02609$	0.0523	No ($p = 0.3185$)	No ($p < 0.0001$)
0006	0.63 ± 0.31	49.7	8[c]	$y = -0.003057x + 0.8009$	0.0830	No ($p = 0.1825$)	No ($p < 0.0001$)
0007	0.69 ± 0.41	59.2	9[d]	$y = 0.007763x + 0.08556$	0.5147	Yes ($p < 0.0001$)	No ($p < 0.0001$)
0011	0.12 ± 0.84	697.5	11	$y = 0.01004x - 0.3681$	0.2898	Yes ($p = 0.0012$)	No ($p < 0.0001$)
0012	0.78 ± 0.28	35.4	11	$y = 0.000917x + 0.7316$	0.0171	No ($p = 0.4680$)	No ($p < 0.0001$)
0013	0.55 ± 0.47	84.6	8[e]	$y = 4.446e^{-005}x + 0.5730$	0.0000	No ($p = 0.9873$)	No ($p < 0.0001$)
0014	1.17 ± 0.25	21.2	8[a,f]	$y = 0.002171x + 1.033$	0.0776	No ($p = 0.1875$)	No ($p < 0.0001$)
0015	− 0.42 ± 0.46	108.3	11[g,h]	$y = 0.003861x - 0.6046$	0.1266	Yes ($p = 0.0457$)	No ($p < 0.0001$)
0016	0.15 ± 0.46	306.1	4[i]	$y = 0.005995x - 0.1866$	0.2522	No ($p = 0.1391$)	No ($p < 0.0001$)
0021	0.75 ± 0.38	50.3%	3	$y = 0.03335x + 0.3425$	0.8996	Yes ($p < 0.0001$)	Yes ($p = 0.2720$)
0023	0.12 ± 0.34	281.4	3[e]	$y = -0.02972x + 0.4873$	0.5975	Yes ($p = 0.0245$)	Yes ($p = 0.5781$)
0024	0.86 ± 0.37	43.4	4[j]	$y = -0.004499x + 1.011$	0.0941	No ($p = 0.3885$)	No ($p = 0.0066$)
0026	0.41 ± 0.19	45.4	6[k]	$y = -0.0008940x + 0.4511$	0.0170	No ($p = 0.6571$)	Yes ($p = 0.2059$)
Cohort	0.53 ± 0.42	139.1% ± 175.7%					

All analyses were performed using $n = 3$ technical replicates unless otherwise noted

All graphs: data are the mean ± SEM of $N = 3$ independent repeat experiments, with $n = 3$–4 technical repeats undertaken per experiment, as cell number permitted. Regressions (solid lines) were calculated taking into account the N and scatter among replicates, with 95% confidence intervals indicated by dotted lines. Where not visible, error bars are smaller than the symbol

[a] $n = 4$ replicates at test 3

[b] $n = 2$ replicates at test 5

[c] $n = 2$ replicates at test 8

[d] n = 2 replicates at test 9

[e] $n = 2$ replicates at test 3 and 5

[f] $n = 2$ replicates at test 4

[g] $n = 4$ replicates at test 7

[h] $n = 2$ replicates at test 8 and 9

[i] $n = 1$ replicate at test 3

[j] $n = 2$ replicates at test 1 and 2

[k] $n = 2$ replicates at tests 1, 2, 5, and 6

(3.8 ± 1.5 nmol mg^{-1} h^{-1}, $n = 19$ [38]). While a direct comparison of these data with our results was not possible due to the differing normalisation factors utilised (mg protein vs. cell number), based on an estimated collection of 1 mg total protein per buccal sample the highest rate of uptake observed in our study (5.0 ± 0.2 nmol mg^{-1} h^{-1}; #0002, day 21) was approximately threefold higher than normal DPD activity in BMC collected from healthy volunteers. This was also 1.3-fold higher than the PBMC DPD activity in the DPD-deficient patients. Formal assessment of the relationship between 5-FU uptake phenotype and metabolic clearance in BMC, and their joint impact on mucosal toxicity, may therefore prove informative.

The dynamic phenotype changes observed in the current work indicate that a single spot test would likely be unsuitable to assess long-term risk of 5-FU induced mucositis in patients, however its utility for the prediction of short-term (current cycle) risk is perhaps worthy of further investigation. Furthermore, this assay represents a simple and minimally invasive technique for investigation of the mechanisms underpinning 5-FU transport and toxicity in normal primary mucosal cells.

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Compliance with ethical standards

Conflict of interest The authors declare no conflicts of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the national research committee (New Zealand Northern A Health and Disability Ethics Committee: 14/NTA/186, 15/NTA/14, 17/NTA/160) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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