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# A novel *in vitro* platform for the study of SN38-induced mucosal damage and the development of TLR4-targeted therapeutic options

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**Abstract**

Tight junction and epithelial barrier disruption is a common trait of many gastrointestinal pathologies, including chemotherapy-induced gut toxicity. Currently, there are no validated *in vitro* models suitable for the study of chemotherapy-induced mucosal damage that allow paralleled functional and structural analyses of tight junction integrity. We therefore aimed to determine if a transparent, polyester membrane insert supports a polarised T84 monolayer with the phenotypically normal tight junctions. T84 cells (passage 5-15) were seeded into either 0.6 $cm^2$, 0.4 μm pore mixed cellulose transwell hanging inserts, or 1.12 $cm^2$ 0.4 μm pore polyester transwell inserts at varying densities. Transepithelial electrical resistance (TEER) was measured daily to assess barrier formation. Immunofluoresence for key tight junction proteins (occludin, zonular occludens-1, claudin-1) and transmission electron microscopy were performed to assess tight junction integrity, organelle distribution and polarity. RT-PCR was performed to determine expression of TLR4. Liquid chromatography was also conducted to assess SN38 degradation in this model. Polyester membrane inserts support a polarised T84 phenotype with functional tight junctions *in vitro.* Transmission electron microscopy indicated polarity, with apico-laterally located tight junctions. Immunofluorescence showed membranous staining for all tight junction proteins. No internalisation was evident. T84 cells expressed TLR4, although this was significantly lower than levels seen in HT29 cells ($p=0.0377$). SN38 underwent more rapid degradation in the presence of cells (-76.04±1.86%) compared to blank membrane (-48.39±4.01%), indicating metabolic processes. Polyester membrane inserts provide a novel platform for paralleled functional and structural analysis of tight junction integrity in T84 monolayers. T84 cells exhibit the unique ability to metabolise SN38 as well as expressing TLR4, making this an excellent platform to study clinically relevant therapeutic interventions for SN38-induced mucosal damage by targeting TLR4.

**Key words:** *in vitro* model, transwell support, barrier function, tight junctions, SN38, toll-like receptor 4

## Introduction

The intestinal epithelium has two important and distinctly different roles within the gastrointestinal tract (GIT). It mediates the complex absorption of nutrients from the intestinal lumen, and simultaneously represents a barrier separating the internal milieu from the outside environment in both an immunologic and metabolic sense (1). In the gut, the luminal surface comes into direct contact with the highest concentrations of bacteria (2), antigens and a host of potentially toxic compounds (3). Consequently, the paradoxical functions of the intestinal barrier are critical in maintaining gastrointestinal health and homeostasis (4).

Tight junctions provide a paracellular barrier that is selectively permeable to ions and macromolecules. The molecular characteristics and functional properties of tight junctions are subject to modification by a variety of cues, both physiological and pathological, highlighting the highly dynamic nature of these structures (5). Consequently, tight junction disruption often leads to the development of a leaky gut (6); a hallmark feature of compromised mucosal barrier function and many pathological states (7). A thorough understanding of tight junction regulation, signaling and modification is therefore critical to determine how they may contribute to disease progression. This holds particularly true for chemotherapy-induced gut toxicity, which is characterised clinically by increased intestinal permeability (8) and tight junction defects (9, 10). Despite molecular disruption to tight junctions being reported, the underlying mechanisms are unclear and difficult to identify in full physiological systems.

Chemotherapy treatment has long been recognised to induce a leaky gut, with recent research suggesting tight junction disruption may contribute to the development of clinically diagnosed diarrhoea through altered leak-flux mechanisms (11). Irinotecan is a chemotherapeutic drug associated with exceptionally high levels of intestinal toxicity. It serves as the water-soluble precursor of the lipophilic metabolite, SN38, which is formed by carboxylesterase-mediated cleavage of the carbamate bond between the camptothecin moiety and the dipiperdino side chain (12). SN38 is approximately 1000 times as potent as irinotecan as an inhibitor of topoisomerase I and its unique hepatobiliary metabolism is responsible for the high levels of intestinal toxicity (13). SN38 is glucoronidated to SN38 glucuronide (SN38G) and detoxified in the liver via conjugation by the uridine-diphospho-glucuronosyl transferase (UGT1A) family, which releases SN38G into the intestines for elimination (14). However, in the intestinal lumen, bacterial β-glucoronidases are able to regenerate SN38 from

SN38G (15). This second pass metabolism is key to the dose-limiting, and clinically diagnosed, diarrhoea associated with irinotecan treatment.

Although our understanding of irinotecan and SN38-induced gastrointestinal toxicity is improving, many studies' mechanistic data are limited to due the difficulties in accessing the GIT. *In vitro* models therefore offer an appealing alternative of studying GI-related pathologies. Epithelial cell lines derived from the intestine can be cultured as monolayers to mimic the intestinal epithelium and provide insight into the physiological characteristics of tight junctions and epithelial barrier function (4). The human colonic epithelial cell line, T84, derived from a colonic carcinoma, is widely used *in vitro* to assess intestinal barrier function and tight junction integrity (16, 17).

T84 cells are typically cultured *in vitro* using a mixed-cellulose membrane, semi-permeable transport system (Figure 1), which readily allows assessment of barrier function. However, these opaque mixed-cellulose membranes, although considered gold-standard, limit downstream imaging techniques such as light or confocal microscopy, critical for a thorough understanding of tight junction signaling and integrity. We therefore aimed to determine if a transparent, polyester membrane insert supports a polarised T84 monolayer with phenotypically normal tight junctions. The long-term goal is for our model to be used for interrogation of complex gastrointestinal physiology under normal and challenged states. If successful, this model will be used to study mechanisms of chemotherapy (specifically irinotecan)-induced mucosal damage in a simple, high-throughput manner. Additionally, this novel platform could be used to assess the efficacy of anti-mucotoxic agents for both preclinical and clinical translation.

## Materials and Methods

### Cell Culture

Cryopreserved T84 cells (passage 5-15) derived from a human colorectal carcinoma were obtained from Culture Collections (Porton Down, UK; 88021101). HT29 cells (passage 5-15), derived from a human colorectal carcinoma with an epithelial phenotype, were kindly provided by Dr J Hardingham (Queen Elizabeth Hospital, South Australia). MCF-7 cells (passage 1-6), derived from a human breast carcinoma were also a kind gift from Professor M Brown (Royal Adelaide Hospital, South Australia). All cells lines retained their original morphology and growth characteristics over the range of passages used (data not shown).

Cells were thawed in a 37ºC water bath and maintained in a 75 $cm^2$ or 150 $cm^2$ sterile cell culture flask (Corning Life Sciences, MA, USA) at 37ºC with 5% $CO_2$. T84 and HT29 cell culture media was Dulbecco's Modified Eagle Medium/Ham's F-12 Nutrient Mixture containing 15 mM HEPES, L-glutamine and sodium bicarbonate (DMEM/F-12; Sigma-Aldrich, Castle Hill, NSW, Australia; D8437) supplemented with 1% penicillin/gentamicin+fungizone, 10% foetal bovine serum and 1mM l-glutamine (complete DMEM). MCF-7 cells were maintained in RMPI media supplemented with 2 mM L-glutamine and 10% FBS. Experimental cell cultures were grown in sterile, multi-well tissue culture plates under identical growth conditions. Cell lines were routinely passaged when culture monolayers reached approximately 80% confluence at subculture ratios between 1:3 and 1:6 in fresh growth medium. Cells were detached by aspirating growth medium, washing with 1X phosphate buffered saline (PBS; pH 7.4) and incubating with 3 ml of trypsin-EDTA for 10 min at 37ºC (0.05% trypsin, 0.53 mM EDTA; Invitrogen, Mulgrave, VIC, Australia). The reaction was then quenched by the addition of growth medium. Cells were centrifuged at 300 g for 5 min, supernatant removed and cells resuspended in fresh, complete DMEM. Cell count were conducted using an automated cell counter (BioRad, NSA, Australia) and were seeded into either 0.6 $cm^2$, 0.4 μm pore mixed cellulose transwell hanging inserts (Invitrogen, VIC, Australia; PIHA01250), or 1.12 $cm^2$ 0.4 μm pore polyester transwell inserts (Corning Life Sciences, MA, USA; CLS3801). T84 cells were seeded at the following densities: 50,000; 100,000; 200,000 and 400,000 cells/$cm^2$ into the apical chamber. Cell culture media in both the apical and basolateral chambers was changed every 48 h. Transepithelial electrical resistance (TEER) was measured daily using am EVOM2 epithelial volt-ohm-meter with chopstick electrodes (World

Precision Instruments, Sarasota, FL, USA) for 1 week during the growth period and area adjusted for analysis using the following formula; *TEER monolayer ($\Omega/cm^2$) = [raw TEER ($\Omega$) – TEER blank ($\Omega$)]/area of membrane ($cm^2$).* All experiments were performed in triplicate and repeated twice.

**Transmission Electron Microscopy**

After determining optimal cell density (100,000 cells/$cm^2$), cells were seeded into both 0.6 $cm^2$, 0.4 μm pore mixed cellulose transwell hanging inserts (Invitrogen, VIC, Australia; PIHA01250), or 1.12 $cm^2$ 0.4 μm pore polyester transwell inserts (Corning Life Sciences, MA, USA; CLS3801) at a density of 100,000 cells/$cm^2$. Cell culture media was changed every 48 hour. TEER was measured daily from day 3 using an EVOM2 epithelial volt-ohm-meter. Once high, stable TEER was achieved monolayers were fixed overnight in 4% paraformaldehyde/1.25% glutaraldehyde (electron microscopy grade) in 1X PBS (4% sucrose; pH 7.2). Monolayers were washed with 1x PBS + 4% sucrose (v/v) before being post-fixed in 2% osmium tetroxide (w/v) for 1 hour. Monolayers were dehydrated through graded ethanols, removed from the transwell support system and mounted in resin before being polymerised at 70$^o$C for 24 hours. 80μm thick sections were cut on a Leica Ultracut S ultramicrotome using a diamond knife. Sections were picked up on 200mesh copper/palladium grids and stained with uranyl acetate and Lead Citrate. Grids were then visualised using the Philips CM200 transmission electron microscope (TEM). Monolayers were assessed for the presence of tight junctions, organelle distribution, polarity and monolayer formation. Monolayers with TEER values over 1000 $\Omega/cm^2$ were used in all experiments.

**Immunofluorescence**

A secondary aim of this study was to determine if the polyester membrane transwell support systems support immunofluorescence and confocal imaging. T84 cells were seeded into 1.12 $cm^2$ 0.4 μm pore polyester transwell inserts at a density of 100,000 cells/$cm^2$. Once T84 monolayers had developed stable TEER values >1000 $\Omega/cm^2$ cell culture media was aspirated and cells washed with ice-cold 1X PBS pH 7.4. A fixing solution (1:1 v/v acetone/methanol stored at -20$^o$C) was applied to the apical chamber (500 μl) for 15 min. Cells were rinsed with 1X PBS and permeabilised using 0.1% (v/v) Triton X-100/PBS for 3 min. After 2 x 5 min washes in 1X PBS, cells were blocked overnight in 3% (w/v) bovine serum albumin (BSA)/PBS at 4$^o$C (Sigma-Aldrich, NSW, Australia; 9048-46-8). The blocking solution was aspirated and 200 μl of primary antibody was applied to the apical chamber

(mouse mAb occludin, Invitrogen 33-1500, 5 μg/ml; rabbit pAb ZO-1, Invitrogen 61-7300, 2.5 μg/ml; rabbit pAb claudin-1, Abcam ab15098, 2 μg/ml). All primary antibodies were diluted in 1% (w/v) BSA/PBS and incubated for 1 h at room temperature. The primary antibody was then aspirated, and cells washed in 1X PBS+0.05% Tween (4 x 5 min). 200 μl of fluorescent-conjugated secondary antibody (anti-mouse 488; anti-rabbit 568 Alexa Fluor®; Invitrogen, VIC, Australia) was added to the apical chamber at 100 μg/ml. All secondary antibodies were diluted in 1% (w/v) BSA/PBS and incubated for 1 hour at room temperature. For nuclear staining, cells were incubated with 1 μg/ml 4',6-diamidino-2-phenylidole (DAPI) for 10 min at room temperature. Cells were washed in 1X PBS+0.05 Tween for 4 x 5 min before the membranes were removed from the inserts and mounted onto glass microscope slides using Fluoroshield™ (Sigma-Aldrich, NSW, Australia; #F6182). Cells were visualised using the SP5 Spectral Scanning Confocal Microscope (Leica, Wetzlar, Germany). Negative controls had the primary antibody omitted.

**Liquid chromatography–mass spectrometry**

The long-term goal is for this *in vitro* model to be used to assess the mechanisms involved in SN38-induced mucosal damage to identify potential targets for the development of interventions. It is well recognised that SN38, the active metabolite of irinotecan, is a basic compound and typically unstable in many physiological solutions. It is therefore important to characterise the degradation and potential metabolism of SN38 in this model prior to implementing its usage. As per section 2.3, cells were seeded in triplicate into polyester membrane transwell support systems and TEER monitored daily. When a stable TEER >1000 $\Omega/cm^2$ was achieved, cells were treated with 5 μM SN38 in the apical and basolateral chambers. To determine the natural degradation of SN38, transwell systems containing no T84 cells were filled with SN38 supplemented cell culture media (5 μM). Each transwell system with T84 cells and without T84 cells (negative control) was subject to identical conditions. 25 μl from the apical and basolateral chambers was collected at 0 h, 1 h, 3 h, 6 h, 24 h and 48 h. Apical and basolateral samples were combined (50 μl) to produce a single sample per time point. All experiments were performed in triplicate and repeated.

*Sample Preparation*

50 µl of sampled cell culture media was added to 150 µl of ice-cold acetonitrile with 0.1% formic acid (containing 20 ng/ml of IS). Samples were vortexed for 10 s and centrifuged at 13,300 rpm for 10 min at room temperature. A 180 µl aliquot of the supernatant was transferred to a clean microtube and 10 µl samples were analysed in triplicate using liquid chromatography–mass spectrometry (ABSCIEX TripleTOF™ 5600 LC/MS/MS).

*Chromatographic Conditions*

The ABSCIEX TripleTOF™ 5600 LC/MS/MS was used to perform liquid chromatography–mass spectrometry analysis. Chromagraphic separation was achieved by using a Kinetex C18 (2.6 µl, 50 mm x 3.0 mm) analytical column (Phenomenex, NSW, Australia; #00A-4633-AN). The mobile phase A was 5% acetonitele, 95% water, 0.1% formic acid. Mobile phase B was 95% acetonitele, 95% water, 0.1% formic acid with a 0.2 ml/min flow rate. The gradient system commenced with 90/10 mobile phase A/B for 30 s, whilst mobile phase A was gradually decreased to 0% by 2 min. This was maintained for 3 min before returning to initial conditions. The retention times for SN38 and camptothecin (CPT; internal standard) were 2.1 min and 2.16 min, respectively.

*Standard Curve Generation*

A working standard solution of 1 µg/ml was diluted accordingly to achieve: 500 ng/ml, 375 ng/ml, 250 ng/ml, 125 ng/ml, 50 ng/ml, 25 ng/ml, 5 ng/ml and 2.5 ng/ml of SN38. 10 µl of each solution were spiked into 40 µl of blank plasma. Final SN38 concentrations were 100 ng/ml, 75 ng/ml, 25 ng/ml, 5 ng/ml, 1 ng/ml and 0.5 ng/ml. The lower limit of detection was 0.1 ng/ml and lower limit of quantification was 0.5 ng/ml. SN38 concentration was determined by interpolation from the calibration curve and presented as molarity and percentage decrease from baseline.

**Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis of TLR4**

Toll-like receptor 4 (TLR4) is an emerging mediator of irinotecan-induced gut toxicity and a promising target for the development of potential interventions. In order for this platform to be used in the development of TLR4-targted interventions, it is imperative that TLR4 expression be confirmed. TLR4 expression in HT29 and Caco-2 cells was used as a positive control.

*RNA Isolation*

To determine TLR4 mRNA expression, T84 (p7), Caco-2 (p46) and HT29 (p5) cells were grown to confluence in 90mm culture dishes. Total RNA extraction was performed on each cell line using the NucleoSpin RNA Isolation Kit as per manufacturer's instructions (NucleoSpin RNA Isolation Kit, Macherey-Nagel, Düren, Germany; #740955). Once eluted, RNA was stored at -80 ºC. Total RNA was quantified using the BioTek Synergy™ Mx Microplate Reader (BioTek, Vermont, USA), TAKE3 plate and Gen5 (version 2.00.18) software. RNA purity was also determined using the the BioTek Synergy™ Mx Microplate Reader (BioTek, Vermont, USA), TAKE3 plate and Gen5 (version 2.00.18) software. RNA integrity was assessed at the Adelaide Microarray Facility (South Australia Health and Medical Research Institute) using the Agilent 2100 Bioanalyser RNA 600 Nano Chip (Series II) kit.

*Reverse Transcription and RT-PCR*

1 µg of total RNA was reverse transcribed using the iScript™ cDNA Synthesis Kit (BioRad, NSA, Australia; #1708890) as per manufacturers instructions. RT-PCR was performed using the Rotor-Gene 3000 (Corbett Research, NSW, Australia). Amplification mixes contained 2 µl of cDNA sample (100 ng/µl), 5 µl of SYBR green fluorescence dye, 2 µl of RNase-free water and 0.5 µl of each forward and reverse primers, prediluted to 50 pmol/µl, to make a total volume of 10 µl. Thermal cycling conditions included a denaturing step at 95 ºC for 15 min, followed by 40 cycles of denaturation at 95ºC for 15s, annealing at 60 ºC for 15s and extension at 72 ºC for 15 s. All samples were run in triplicate. Primer efficiency was evaluated using standard curves and experimental threshold (Ct) values were calculated by the Rotor Gene 6 programme. $C_T$ values were used to quantify relative mRNA expression of TLR4 in T84 cells and human colonic tissue using the $\Delta C_T$ method, relative where TLR4 expression = $2^{-[C_T(target)-C_T(reference)}$. β-actin was used as an internal housekeeping (reference) gene.

Primer details are as follows: TLR4 primer sequence 5'-3' F: TGA GCA GTC GTG CTG GTA TC ($Tm^3$ 54ºC), R: CAG GGC TTT TCT GAG TCG TC ($Tm^3$ 54ºC), NM_003266.3, 167 bp. β-actin primer sequence 5'-3' F: CTC TTC CAG CCT TCC TTC CT ($Tm^3$ 54ºC), R: AGC ACT GTG TTG GCG TAC AG ($Tm^3$ 54ºC), NM_001101.3, 116 bp.

## Statistical analysis

Data were compared using Prism version 7.0 (GraphPad® Software, San Diego, USA). A D'Agostino-Pearson omnibus test was used to assess normality. When normality was confirmed, a paired t-test or two-way analysis of variance with appropriate post hoc testing were performed to identify statistical significance. In other cases, a Kruskall-Wallis test with Dunn's multiple comparisons test and Bonferroni correction was performed. A p-value of $<0.05$ was considered significant.

***Table 2*** Primer Sequences and Characteristics

| Gene | Primer Sequence (5'-3') | mRNA Sequence | Amplicon Length | Tm[3] (ºC) |
|---|---|---|---|---|
| TLR4 | F: TGA GCA GTC GTG CTG GTA TC | NM_003266.3 | 167 bp | F: 54 |
| | R: CAG GGC TTT TCT GAG TCG TC | | | R: 54 |
| β-actin | F: CTC TTC CAG CCT TCC TTC CT | NM_001101.3 | 116 bp | F: 54 |
| | R: AGC ACT GTG TTG GCG TAC AG | | | R: 54 |

** Designed by Primer3, analysed using NetPrimer software and synthesised by Geneworks Ltd.*

## Results

*Polyester membrane inserts support a polarised T84 phenotype with functional tight junctions in vitro*

T84 cells seeded at all density ranges, in both transwell support systems, achieved TEER values greater than 1000 Ω/cm$^2$ within seven days (Figure 2a, 2b). T84 cells seeded at ≥200,000 cells/cm$^2$ had an elevated trajectory, whilst a seeding density of 50,000 cells/cm$^2$ displayed delayed maturation and resistance development. Monolayers seeded at 100,000 cells/cm$^2$ displayed consistent and controlled development of resistance. This trajectory is likely to facilitate optimal differentiation. When seeded at 100,000 cells/cm$^2$, T84 cells exhibited comparable resistance development characteristics in both transwell support systems, with no statistically significant differences observed (Figure 2c).

TEM analysis confirmed T84 monolayers grown on polyester membranes displayed polarity, with basally-located nuclei and apical microvilli (Figure 3a). TEM analysis also showed intercellular junctional complexes (tight junction, intermediate junction and desmosome) at the apico-lateral border (9) of cells (Figure 3b, 3c). The presence of molecularly intact tight junctions in polyester membrane support system was confirmed by immunofluorescence for three major tight junction proteins: zonular occludens-1 (ZO-1), occludin and claudin-1 (Figure 4a-c). No internalization or cytoplasmic redistribution was evident. These results also highlight the imaging potential of this support system, facilitating both light and confocal microscopy techniques. No fluorescence was detected on any membrane that had the primary antibody omitted (data not shown).

*SN38 stability in the transwell support system*

SN38 underwent rapid degradation in cell culture media alone (negative control; -T84 monolayer), with a 48.39±4.01% decrease from baseline by 1 h (Figure 5a, 5b). This decline was more pronounced in transwell systems with T84 monolayers (-76.04±1.86%) indicating metabolic processes (*$p<0.0001$; $p=0.0126$). SN38 concentration plateaued between 1 h and 24 h, although a small increase was observed at 48 h in the presence of T84 monolayers suggesting possible efflux mechanisms.

*T84 cells express TLR4*

RNA integrity numbers (RIN) were assigned to each sample using the Agilent Bioanalyser. Caco-2 cell yielded the highest RIN score (RIN: 8.90). HT29 and T84 cells had RIN scores of 8.00 and 7.90

respectively. RNA $A_{260/280}$ ratios for Caco-2, HT29 and T84 cells were 2.098, 2.089 and 2.090, respectively, indicating pure, protein-free samples.

Under basal conditions, T84 cells expressed TLR4 (Figure 6), although this was significantly less than that of the HT29 cells (*p=0.037).

## Discussion

This study has clearly highlighted the potential application for clear, polyester membrane transwell support systems in the investigation of gastrointestinal pathology. We have shown comparable T84 epithelial cell growth patterns in this model and the traditionally used, gold-standard mixed-cellulose membranes. T84 cells display normal morphological features when grown on polyester membranes, with a polarised phenotype complete with apical microvilli and apico-lateral tight junctions. We intend for this model to be used for side-by-side structural and functional analysis of tight junctions and their contribution to the development of chemotherapy-induced gastrointestinal dysfunction. Correspondingly, we have characterised SN38 metabolism in this model, highlighting the metabolic capabilities of T84 cells. We have also confirmed expression of TLR4, a key mediator of toxicity and promising target for therapeutic interventions.

Given the inherent challenges in accessing the gastrointestinal tract, a simple *in vitro* model for interrogation of complex gastrointestinal physiology is critical in unraveling the mechanisms of chemotherapy-induced diarrhoea. Characterisation of this model highlights its suitability for the study for SN38-iduced mucosal damage and the mechanisms and/or implications for tight junction disruption. Ultrastructurally, these monolayers displayed normal characteristics of the intestinal epithelium, with a microvillus-studded apical membrane, polarity and typical organelle distribution, supporting previous phenotype reports (16). Most importantly, intercellular junction complexes were evident and apical tight junctions appeared phenotypically normal displaying apposing leaflets. Molecularly, tight junctions of polyester membrane T84 monolayers expressed the key architectural proteins, ZO-1, claudin-1 and occludin which exhibited typical ‘honeycomb’ or ‘cobblestone’ distribution.

It is well documented that tight junctions undergo an array of molecular changes in response to pathological cues (18) with post-translational degradation (19) and redistribution/internalisation of tight junction proteins reported in response to inflammatory mediators (20-22), kinases (23) and microbiota changes (24, 25). Internalisation is described most commonly for ZO-1, as it resides in the cytoplasm adjacent to the plasma membrane of the cell. It has been reported that under pathological states, particularly those with an inflammatory component, ZO-1 can detach from the junctional complex leading to altered tight junction integrity and barrier disruption (26). There was no evident

internalisation of ZO-1, claudin-1 or occludin in T84 monolayers grown in polyester membrane support systems, indicating that this systems supports molecularly intact, functional tight junctions that resemble that of the GIT. Furthermore, the transparent properties of the polyester membranes enable powerful confocal and light microscopy, enhancing the structural analysis of these complex intercellular structures and allowing paralleled functional and structural analyses. This feature is a significant advantage of this *in vitro* model, as structural tight junction analysis is typically achieved by growing T84 cells on coverslips. The polyester membrane transwell support system therefore provides a novel platform for interrogation of complex gastrointestinal physiology under normal or challenged situations and will enable investigation of SN38-induced mucosal damage.

SN38 is the semi-synthetic analogue of the naturally occurring anticancer alkaloid camptothecins and the active metabolite of irinotecan. The hydroxyl group at the $C_{10}$ position and ethyl group at the $C_7$ position both help to stabilise SN38 in physiological environments and thus improve its potency (27). Despite this, SN38 is poorly solubilised and remains relatively unstable in physiological solutions. For example, the lactone ring of SN38 is stable at pH $\leq$ 4.5 but hydrolyses completely to its carboxylated form at pH $\geq$ 9. At pH 6.7, both forms are in equilibrium. In our *in vitro* T84 transwell model, SN38 underwent rapid degradation in physiologically stable cell culture media alone (negative control) highlighting not only the instability of SN38, but also its relatively short half-life. In the presence of T84 cells, this degradation was more pronounced, with a further 27.65% reduction from baseline suggesting T84 cells express the enzymes required for SN38 metabolism (e.g. UGT1A) and further highlights the suitability of this model for the study of SN38-induced mucosal damage. In addition to metabolism, our results also showed evidence of SN38 efflux at 48 hours, indicating possible expression of efflux transporters that utilise SN38 as a substrate in this T84 cell line. ATP binding cassette (ABC) transporters have been characterised in other human colorectal carcinoma derived cell lines, such as LS513 (28), but their expression in T84 cells is unclear. The ABC transporter, multidrug resistance gene 1 (MDR1), as well as and cytochrome P450 isoform 3A4 (CYP3A4), have also been detected in LS180 cells, whereas only CYP3A4 was inducible in Caco-2 cells and TC-7 cells (29). Naruhashi and colleagues (2011) have recently shown that mRNA expression the ABC transporters MDR1, multidrug resistance-associated protein (MRP) 2 and MRP3 is comparable between caco-2 lines and T84 monolayers (30), with minimal differences their pattern of change in response to various endogenous compounds and xenobiotics. Despite similar expression, the intrinsic function of MDR1

was stronger in caco-2 cells suggesting caco-2 models are more suitable for the study of drug transport. Despite this, T84 cells were found to be more sensitive to stimulation by these endogenous compounds/xenobiotics presumably due to their relatively undifferentiated state relative to caco-2 monolayers. T84 cells therefore present as a superior model for assessing the induction capacity of compounds and mechanisms of gastrointestinal pathology, whilst caco-2 models are advantageous in the analysis of drug transport mechanisms and pro-drug development. We also showed that T84 cell lines express TLR4, whereas the caco-2 cell line did not. Although this largely does not support the literature, it has been reported that some caco-2 cells will only express TLR4 when stimulated with LPS (31). In fact, TLR4 expression is comparatively lower in caco-2 cell lines when compared with numerous other colonic cell lines such as HT29s and T84s (31). This is also the case for TLR4 expression in T84 cells, which in our study was comparatively lower than that seen in HT29 cells. Nonetheless, the current study has definitively shown that T84 cells express TLR4, thus supporting the use of this novel *in vitro* platform for the study of TLR4-targeted interventions for chemotherapy-induced gut toxicity. Development of this *in vitro* model would therefore not only provide insight into the mechanisms of SN38-induced mucosal damage, but would also provide an excellent platform in which to investigate pharmacological strategies critical in the development of effective treatment strategies.

Given the inherent challenges in accessing the gastrointestinal tract, the study of chemotherapy-induced gut toxicity remains difficult. A simple model for interrogation of complex gastrointestinal physiology is therefore critical in unraveling the mechanisms of symptoms such as diarrhoea. This study has successfully demonstrated the use of T84 cells, grown in transparent polyester transwell support systems, as a suitable model for the study of chemotherapy-induced mucosal damage. This platform supports a polarised T84 phenotype with functional tight junctions, allowing for in-depth permeability studies. Additionally, the transparent properties of these inserts allows for sophisticated downstream analyses such as live-cell fluorescent imaging or confocal microscopy. We also demonstrated that T84 cells exhibit the unique ability to metabolise SN38 as well as expressing TLR4, making this an excellent platform for the study of clinically relevant therapeutic interventions for SN38-induced mucosal damage by targeting TLR4.

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**Compliance with ethical standards**

**ALL** authors declare they have no conflicts of interest.

**Ethical approval:** This article does not contain any studies with human participants or animals performed by any of the authors.

**Author contributions**

All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript; HRW, KRS, YZAVS conducted the experiments; HRW, JMB, RJG, RML designed the model; HRW, YZAVS, RJG, JMB wrote the manuscript.

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**Figure legends**

***Figure 1*** **(A) Permeable transport support system.** This system consists of an inner chamber allowing the T84 monolayer to be suspended in a supportive media (DMEMHam's/F12+1%penicillin/gentamicin+fungizone; 1mM L-glutamine; 10%FBS). **(B) Electric volt ohm-meter.** The electrodes of an electric volt ohm-meter pass current and measure voltage to determine the transepithelial resistance of the monolayer (ohms). For reproducible results, the position and stability of the electrodes must remain consistent.

***Figure 2*** **Transepithelial resistance (TEER) development over seven days in T84 cells in (A) mixed cellulose membrane support systems and, (B) polyester membrane support systems.** All seeding densities achieved resistance in both membrane types within seven days. T84 cells seeded at ≥200,000 cells/$cm^2$ had an elevated trajectory, whilst a seeding density of 50,000 cells/$cm^2$ displayed delayed maturation and resistance development. **(C)** Optimal seeding density appears to be 100,000 cells/$cm^2$ with monolayers showing comparable resistance development in both support systems ($p>0.05$). TEER was measured using an EVOM2 epithelial volt-ohm-meter (Physiologic Instruments). Data expressed as mean±SEM and analysed using a one-way analysis of variance with Tukey's post hoc.

***Figure 3*** **Transmission electron microscopy images of T84 cells grown on polyester membrane hanging inserts.** Panel A (4200x) shows cellular polarity with apical microvilli (arrow) and a basally-located nucleus (N). Panel B (16500x) shows intercellular junction complexes at the apico-lateral boundary. Panel C (26500x) shows high power image of the junctional complex; TJ = tight junction, IJ = intermediate junction, D = desmosome.

***Figure 4*** **Immunofluorescent staining of (A) ZO-1 (red), (B) occludin (green) and (C) claudin-1 (cyan) in T84 monolayers.** The border of each cell can be distinguished by immunocytochemical circumferential staining of each tight junction protein. T84 monolayers were fixed in 1:1 (v/v) acetone/methanol, before being permeabilised with 0.1% TritonX-100. Cells were blocked with 3% BSA before the primary antibodies for ZO-1, occludin and claudin-1 were applied. Monolayers were then incubated with fluorescently-conjugated secondary anti-rabbit/mouse antibodies at 568 nm and 488 nm, respectively. Panel C has been pseudocoloured cyan for this figure. **(D) XZ image showing occludin staining (arrow) at the cell periphery**. The composite XZ stack shows apically located occludin staining which ceases toward the basal surface of the cell. The XZ composite was generated from 30 x 1 μm z-sections using the public domain Java image processing programme, Image J.

***Figure 5*** **SN38 concentration in transwell support system±T84 monolayers.** Data presented as **(A)** concentration (nM) or, **(B)** relative to baseline (%). Transwell support systems±T84 monolayers were treated with 5 μM SN38. The apical and basolateral chambers were sampled at 1 h, 3 h, 6 h, 24 h and 48 h. SN38 concentration was determined by liquid chromatography–mass spectrometry (ABSCIEX TripleTOF™ 5600 LC/MS/MS). SN38 underwent rapid degradation in cell culture media, with a 48.39±4.01% decrease from baseline by 1 h. Degradation is more pronounced in transwell supports with T84 monolayers suggesting metabolic processes (*$p<0.0001$; □$p=0.0126$). Possible efflux of SN38 can be seen at 48 h, with a spike in concentration. This is only observed in the presence of T84 monolayers.

***Figure 6*** **TLR4 mRNA expression in T84 and HT29 cells relative to internal housekeeping gene β-actin.** Relative expression was determined using the $\Delta C_T$ method. Expression of TLR4 in Caco-2 cells was below detectable levels. Data expressed as mean±SEM. A one-way analysis of variance with Tukey's post hoc was performed to dertermine statistical significance.