



# Microbial sophorolipids inhibit colorectal tumour cell growth in vitro and restore haematocrit in Apc min+/- mice

Breedge Callaghan, Matthew Twigg, Niki Baccile, Inge van Bogaert, Roger Marchant, Christopher Mitchell, Ibrahim Banat

## ► To cite this version:

Breedge Callaghan, Matthew Twigg, Niki Baccile, Inge van Bogaert, Roger Marchant, et al.. Microbial sophorolipids inhibit colorectal tumour cell growth in vitro and restore haematocrit in Apc min+/- mice. *Applied Microbiology and Biotechnology*, 2022, 106 (18), pp.6003-6016. 10.1007/s00253-022-12115-6 . hal-03911204

**HAL Id: hal-03911204**

**<https://hal.science/hal-03911204>**

Submitted on 22 Dec 2022

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

[Click here to view linked References](#)

**Microbial sophorolipids inhibit colorectal tumour cell growth *in vitro* and restore haematocrit in Apc<sup>min+/-</sup> mice**

Breedge Callaghan<sup>1</sup>, Matthew S. Twigg<sup>1</sup>, Niki Baccile<sup>2</sup>, Inge N. A. Van Bogaert<sup>3</sup>, Roger Marchant<sup>1</sup>, Christopher A. Mitchell<sup>1,\*</sup> & Ibrahim M. Banat<sup>1,\*</sup>

1. School of Biomedical Sciences, Ulster University, Coleraine, BT52 1SA, U.K.

2. Sorbonne Universités, UPMC Univ Paris 06, CNRS, Collège de France UMR 7574, Chimie de la Matière Condensée de Paris, UMR 7574, 75005 Paris, France.

3. Centre for Synthetic Biology, Department of Biotechnology, Ghent University, Coupure Links 653, 9000 Ghent, Belgium.

BC : ORCID - 0000-0002-0798-2540

MST : ORCID - 0000-0002-8601-1960

NB : ORCID - 0000-0003-3142-3228

INAVB : ORCID - 0000-0001-8144-6466

RM : ORCID - 0000-0001-5213-229X

IMB : ORCID - 0000-0001-8964-2840

CM : ORCID - 0000-0002-4422-7499

\*Corresponding Authors – Ibrahim Banat and Christopher Mitchell

Email: im.banat@ulster.ac.uk, ca.mitchell@ulster.ac.uk

Tel: +44 28 7012 3062, +44 28 7012 4089

**Acknowledgments**

The authors would like to thank the staff at the Biomedical and Behavioural Research Unit at Ulster University for their technical assistance with the *in vivo* aspects of this study. This research was funded by both the European FP7 Project grant number 289219 “BIOSURFING” and Invest Northern Ireland Proof of Concept grant number 826.

## Abstract

Sophorolipids are glycolipid biosurfactants consisting of a carbohydrate sophorose head with a fatty acid tail and exist in either an acidic or lactonic form. Sophorolipids are gaining interest as potential cancer chemotherapeutics due to their inhibitory effects on a range of tumour cell lines. Currently, most anti-cancer studies reporting the effects of sophorolipids have focused on lactonic preparations with the effects of acidic sophorolipids yet to be elucidated. We produced a 94% pure acidic sophorolipid preparation which proved to be non-toxic to normal human colonic and lung cells. In contrast we observed a dose-dependent reduction in viability of colorectal cancer lines treated with the same preparation. Acidic sophorolipids induced apoptosis and necrosis, reduced migration and inhibited colony formation in all cancer cell lines tested. Furthermore, oral administration of 50 mg kg<sup>-1</sup> acidic sophorolipids over 70 days to Apc<sup>min/+</sup> mice was well tolerated and resulted in an increased haematocrit, as well as reducing splenic size and red pulp area. Oral feeding did not affect tumour numbers or sizes in this model. This is the first study to show that acidic sophorolipids dose-dependently and specifically reduces colon cancer cell viability in addition to reducing tumour-associated bleeding in the Apc<sup>min/+</sup> mouse model.

**Keywords:** Sophorolipids, Acidic, Colorectal, Cancer, Apc<sup>min/+</sup>

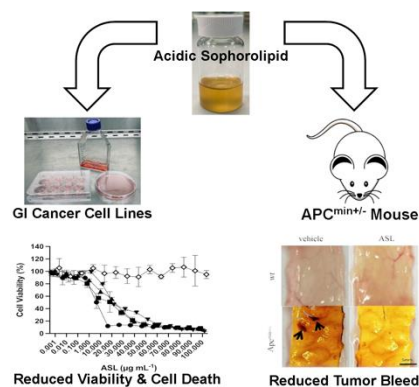
## Statements and Declarations

Competing interests: The authors declare that they have no competing interests.

Ethical approval: The article does not contain any studies with human participants where ethical was required. Experiments using animals were carried out after obtaining ethical approval and under licence in accordance with the Animals (Scientific Procedures) Act 1986, U.K. Further detail is provided in the material and methods section.

Data availability statement: The datasets generated during and/or analysed during the current study are available from the corresponding authors on reasonable request.

## Graphical Abstract



## Key Points

Acidic sophorolipids are produced by yeast species such as *Starmerella bombicola*.

Acidic sophorolipids selectively killed colorectal cells with no effect on healthy gut epithelia.

Acidic sophorolipids reduced tumour associated gut bleed in a colorectal mouse model.

## Introduction

Familial Adenomatous Polyposis (FAP) is an autosomal dominant hereditary form of Colorectal cancer was responsible for over 10% of global cancer incidence and 9.4% of global cancer related deaths in 2020 (Ferlay et al. 2020). FAP is characterised by the development of numerous adenomas along the colorectal tract. Currently, the gold standard for treatment of FAP is surgery, followed by adjuvant chemotherapy (O'Connell et al. 2010). Adjuvant chemotherapy does not however discriminate between normal and transformed tissue, which leads to a variety of potentially serious complications including cardiotoxicity, immune dilapidation, and neurotoxicity (Morgan and Rubin 1998; Mazevet et al. 2013). To allay this problem, compounds that are non-toxic, orally tolerated and specifically target epithelial neoplastic cells in the intestinal tract could have great potential in delaying progression of intestinal neoplasms; in particular, those that are associated with progression to these colorectal cancers (Kelloff et al. 1994; D'Incalci et al. 2005).

There is an increasing body of research demonstrating compounds derived from natural sources such as plants and microbial secondary metabolites have preventative or targeted anti-cancer activity (Fridlender et al. 2015; Khalifa et al. 2019). An example that is already in widespread in cancer treatment is Paclitaxel, first derived from the bark of the yew tree (*Taxus baccata*) (Stierle et al. 1993) and is now used for the treatment of several different cancer types including ovarian, oesophageal, breast, lung and pancreatic cancer (Barbuti and Chen 2015).

Sophorolipids are a class of naturally occurring glycolipids produced by yeast species such as *Starmerella bombicola* (Van Bogaert et al. 2007; Roelants et al. 2019). Comprising a hydrophilic sophorose moiety covalently linked to hydrophobic hydroxylated fatty acid tails ranging between 16 and 18 carbons in length, sophorolipid congeners are produced in two main forms either lactonic (LSL) or acidic (ASL) (Fig. S1) (Banat et al. 2010; Marchant and Banat 2012a; Marchant and Banat 2012b). During fermentation yeast species generate mixtures of these structurally different congeners (Van Bogaert *et al.*, 2007). The anti-cancer properties of sophorolipids have received a lot of attention in recent years. Sophorolipids and their synthetically synthesised derivatives have shown *in vitro* cytotoxic effects in human pancreatic (HPAC), liver (H7402), lung (A549), brain (LN229, HNCG-2), oesophageal (KYSE109, KYSE450), breast, cervivcal (HeLa), leukaemic (HL60, K562) and melanoma (SK-MEL-28) cell lines (Chen et al. 2006a; Chen et al. 2006b; Fu et al. 2008; Dhar et al. 2011; Shao et al. 2012; Ribeiro et al. 2015; Li et al. 2017; Ceresa et al. 2021; Adu et al. 2022). Sophorolipids have also shown potential tumour shrinking capability in an *in vivo* model of cervical cancer (Li et al. 2017). However, to date, very few *in vivo* bioactivity studies have been reported for sophorolipid mixtures. A small number of toxicology experiments have shown that sophorolipids are non-irritating when topically applied to the skin and eyes of rabbits and non-toxic when administered orally to either mice or rats (Ikeda et al. 1986; Callaghan et al. 2016). Sophorolipid mixtures have also been observed to reduce inflammation and reduce mortality rates in a rat model of severe abdominal sepsis as well as decreasing IgE levels in a murine asthma model (Hardin et al. 2007; Bluth et al. 2008).

Although sophorolipids show promising anti-cancer activity *in vitro*, the gross composition and percentage of congeners within the sophorolipid mixture used in most investigations are not fully disclosed. It can be assumed that LSL enriched preparations are more common, due to the preponderance of LSL's in comparison to ASL's within naturally occurring sophorolipid mixtures (Van Bogaert et al. 2011; Marchant and Banat 2012a). The composition of sophorolipid preparations is an important factor to consider when investigating the targeted or preventative anti-cancer effect of these molecules. The reasoning for this is twofold; firstly, to reduce toxicity resulting from contaminants in crude preparations and, natural products being investigated must be composed completely of highly purified biologically active agent (Beutler 2019; Adu et al. 2022). In the case of pactitaxel and other naturally derived agents, this has necessitated the synthetic production in the laboratory of the active agent with targeted anti-cancer properties (Flam 1994). Secondly, and arguably more important, there is significant difference in the functional properties attributed to LSL and ASL congeners. LSL's have strong antimicrobial action that is not seen in ASL's (Van Bogaert et al. 2007; Elshikh et al. 2017). Previously, we have shown that a highly purified preparation of LSL results in exacerbation of adenomatous tumour growth in the

intestinal tract of a colorectal cancer murine model, (Apc<sup>min/+</sup> mouse), with secondary consequences including splenomegaly and reduced haematocrit levels (Callaghan et al. 2016).

To date, the anti-cancer bioactivity of purified ASL's have only been investigated within melanoma cell lines (Adu et al. 2022). In contrast to our previous work with LSL, an ASL specific targeted anti-cancer effect would be advantageous for several reasons. In comparison to LSL congeners, ASL's have reduced production costs as they are the first congeners to be produced in the bioreactor when less favourable conditions that require a reduced energy requirement are present (Casas and García-Ochoa 1999; Sarubbo et al. 2022). The proportion of ASL within enriched preparations can be improved when the medium is supplemented with polyunsaturated fatty acids and this can be further increased via simple hydrolysis of the sophorolipid mixtures which increases the number of open ring structures (Van Bogaert et al. 2011). Alternatively, a modified strain lacking the lactonising enzyme can be applied. Yet, in contrast to the hydrolysis strategy, acetylation of the glucose moieties is still possible (Roelants *et al.*, 2016). ASL's have enhanced solubility when compared to other sophorolipid congeners, which may prove useful for their use as pharmaceuticals, as they can be dissolved in saline instead of potentially toxic solvents such as DMSO (Baccile et al. 2016). Both the solubility as well as enhanced foaming attributes of ASL's contribute to their preferred use in the food, cosmetic and the bioremediation industries (Roelants et al. 2016; Naughton et al. 2019; Adu et al. 2020).

In this study we investigated the targeted therapeutic effect of purified ASL *in vitro* in human colorectal cancer cell lines and their ability to inhibit tumour growth in Apc<sup>min/+</sup> mice. The Apc<sup>min/+</sup> mouse model of FAP was used as it recapitulates key pathological features of the human disease and provides a useful tool to investigate the effects of genetics, diet, and therapeutic drugs on tumorigenesis in the gastrointestinal tract (Hinoi et al. 2007). Like human FAP, it was first noted that Apc<sup>min/+</sup> mice develop adult-onset anaemia with haematocrit levels <45%, the passage of bloody stools, and have a reduced life span (Moser et al. 1995). Additionally, post-mortem analysis revealed the growth of numerous tumours along the small intestine (Moser et al. 1995). The Apc<sup>min/+</sup> mouse model has also been widely used to test the effects of chemotherapeutics on tumour growth and development such as NSAIDs (Aspirin) (Reuter et al. 2002). In addition, the Apc<sup>min/+</sup> mouse model has also been used to develop a further understanding of clinically used chemotherapeutic drugs such as 5-fluorouracil, which is widely used in the treatment of CRC (Tucker et al. 2002). Therefore, we hypothesize that a highly purified and well characterised preparation of ASL (94% pure diacetylated) will selectively inhibit colorectal tumour growth *in vitro* and delay disease progression in the Apc<sup>min/+</sup> mouse model.

## Material and Methods

ASL production and purification.

ASL's used in this study were generated in house from a crude precursor sophorolipid mixture, (*Sopholiance S*, Batch N°11103A), purchased from *Givaudan* (Vernier, Switzerland). ASL congeners were obtained according to a method described by Baccile *et al.* (2013). In brief, sophorolipids were purified from *Sopholiance S* via liquid phase extraction in acidified ethyl acetate followed by hexane washing. Following purification, the sophorolipid material was subjected to alkaline hydrolysis by diluting in 5 M NaOH (*Merck*) and heating under reflux to 90°C for 10 min. The material was then acidified to pH 4 using 18.5% (w/v) HCl (*Merck*). ASL was then recovered via precipitation in a pentanol / hexane medium (*Merck*) at -18°C (Baccile *et al.* 2013). Following production and purification, SL congeners were identified by a UHPLC system with RS Diode Array detector (*ThermoFisher Scientific*) in conjunction with the amaZon SL dual funnel Ion Trap spectrometer LCMS system (*Bruker*). The percentage relative amounts of each congener were calculated and a ratio of ASL to LSL determined (Smyth *et al.*, 2010).

Cell culture

Colorectal cancer cell lines HT29 (ATCC® HTB-38), HT115 (ECACC 85061104), HCT116 (ATCC® CCL-247), and Caco2 (ATCC® HTB-37) as well as colonic epithelium CCD-841-CoN (ATCC® CRL-1790) cell lines were used in this study. Cell lines were maintained in DMEM low glucose media or MEM low glucose media (*ThermoFisher Scientific*) supplemented with 10% (v/v) foetal bovine serum (*ThermoFisher Scientific*). All cultures were maintained at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>.

Cell viability assay

A total of 1 x 10<sup>4</sup> cells per well were seeded in to a 96 well plate (*Sarstedt*) and allowed to attach overnight before being serum starved for 24 hours. Cells were then treated with concentrations of ASL ranging between 0.001 µg mL<sup>-1</sup> – 100 µg mL<sup>-1</sup> or vehicle-only control (PBS) for 24 hours. Subsequently, 10 µL of a 25 mg mL<sup>-1</sup> solution of MTT (3-(4, 5-dimethylthiazol-2-yl) -2, 5-diphenyltetrazolium bromide) (*Merck*) was added to each well and the

plate further incubated for 1 hour at 37°C. Formazan crystals were solubilized with 100 µL of DMSO (*Merck*). Absorbance at 570 nm was measured using a FLUOstar Omega microplate reader (*BMG-LABTECH*). Experiments were repeated three times with six internal replicates per treatment group. Data are representative of the three independent repeats and presented as mean percentage reduction in absorbance in comparison to vehicle-only controls ± standard deviation (SD).

#### Visualisation of morphological changes induced by ASL treatment

A total of  $1 \times 10^4$  cells per well were seeded on to a 96 well plate (*Sarstedt*) and allowed to attach overnight forming a monolayer before being serum starved for 24 hours. Cells were then treated with both 20 and 70 µg mL<sup>-1</sup> of ASL or vehicle-only control (PBS) for 24 hours. The cells were subsequently imaged with an Axio Scope 1 microscope (*Zeiss*) at 200 × magnification. Images obtained from three random fields were selected and assessed for morphological changes by comparing each cell line to the vehicle-only control, looking for changes in cell shape and confluency. Experiments were plated in triplicate and repeated three times.

#### Quantification of detached cells

A total of  $1 \times 10^4$  cells per well were seeded on to a 96 well plate (*Sarstedt*) and allowed to attach overnight forming a monolayer before being serum starved for 24 hours. Cells were then treated with both 40 and 70 µg mL<sup>-1</sup> of ASL or vehicle-only control (PBS) for 24 hours. 10 µg of supernatant was removed from each well, placed into a 1.5 mL microtube (*Sarstedt*). Cells were stained with 5 µM of both Syto 9 and propidium iodide (*Merck*) by adding directly into the microtube and incubating at room temperature for 30 minutes. The cells were then centrifuged at  $150 \times g$  for 5 minutes and the supernatant was aspirated. The remaining cells pellet was washed using ice-cold PBS (pH 7.4) (*ThermoFisher Scientific*) and spun onto a microscope slide using a Shandon cytocentrifuge (*ThermoFisher Scientific*) for five minutes at  $176 \times g$ . Slides were subsequently imaged with a Axio Scope 1 microscope (*Zeiss*) at 400 × magnification. Following staining with Syto9 and propidium iodide, fluorescent staining was present in nucleus therefore live cells appeared green while dead cells fluoresced red (Altman et al. 1993) and as such cells were counted. A minimum of 1 cell and a maximum of 250 cells were counted per field of view. Experiments were plated in triplicate and repeated three times, data set shown as mean ± Standard Error of the Mean (SEM).



## Acridine orange / ethidium bromide staining and quantification

To determine the mechanism of cell death induced by addition of ASL, cells were stained *in situ* with acridine orange at 10 mg mL<sup>-1</sup> (*Merck*) and ethidium bromide 1 mg mL<sup>-1</sup> (*Merck*). Morphological changes were assessed by fluorescence microscopy. For assessment of apoptosis, a total of  $3 \times 10^4$  cells were seeded onto a 10 mm coverslip (*Agar Scientific*) placed within a 6 well plate and the cells were incubated overnight to form a confluent monolayer. Following serum starvation for 24 hours; ASL (at a concentration of either 20 µg mL<sup>-1</sup> or 70 µg mL<sup>-1</sup>), vehicle control (PBS), or 5 µM of etoposide (apoptotic control) (*Merck*) was added and the plate incubated for a further 24 hours. To determine the number of live cells remaining on the coverslip, the samples were washed three times with ice-cold phosphate buffered saline at pH7.4 (*ThermoFisher Scientific*), followed by incubation with 10 µL of a solution of acridine orange / ethidium bromide with a volumetric ratio of 1:1 for 5 minutes. Finally, the cells were washed again three times with ice-cold PBS at pH7.4 (*ThermoFisher Scientific*). Cells were subsequently imaged with a Axio Scope 1 fluorescence microscope (*Zeiss*) at 400 × magnification. A total of 300 attached cells/coverslip were morphologically identified and counted as necrotic (red/orange nuclei), apoptotic (green condensed or fragmented nuclei) or live (green non-condensed ovoid or rounded nuclei). The operator was blinded to the experimental groups and random fields were selected. Each experiment was replicated three times with six internal repeats per group. Data are representative of three independent repeats and presented as mean ± SEM.

## Scratch assay

For wound healing scratch assays,  $1.6 \times 10^6$  cells were plated in each well of a 6 well plate (*Sarstedt*) and allowed to attach overnight. Cells were serum starved for 24 hours and treated with 5 µg mL<sup>-1</sup> of mitomycin C (*Merck*) for 2 hours prior to the scratch to inhibit proliferation. The “wound” was made by scratching a line in the centre of the confluent monolayer using a sterile toothpick. Cells were rinsed very gently three times with PBS at pH7.4 (*ThermoFisher Scientific*) and cultivated in serum free media supplemented with PBS vehicle-control or 10 - 20 µg mL<sup>-1</sup> ASL for up to 72 hours. The cell was imaged at various time points using a ELWD TI SCP microscope (*Nikon Europe B. V.*) at 100 × magnification. To quantify migration of cells into the scratch wound, the area of the gap was measured using ImageJ software (Schneider et al. 2012). After 72 hours, the area of the remaining

gap was measured and the difference between initial and final areas calculated. Experiments were repeated three times using triplicate technical replicates per experimental group. Data are representative of the three independent repeats and presented as mean  $\pm$  SEM.

#### Chemotactic cell migration assays

Chemotactic cell migration assays were carried out in a modified Boyden chamber, based on a previously described protocol (Chen 2005).  $1 \times 10^4$  cells per well of CCD-841-CoN, HT29 or HT115 CRC cells were placed in the upper compartment of a 96- well FluoroBlok transwell inserts (*Analab*) which contained 8  $\mu$ m pore size polyethylene terephthalate filters (*Analab*) and the cells were allowed to attach overnight. A final concentration of 0, 10 or 50  $\mu$ g mL<sup>-1</sup> of ASL in serum free media was added to the upper chamber, while appropriate culture media supplemented with 10% FBS was added (as a chemoattractant) to the bottom chamber. Cells were left to migrate overnight in an incubator at 37°C and at 5% CO<sub>2</sub>. Following incubation, all media in the upper chamber was removed and all cells that failed to migrate were removed using a sterile cotton swab. Cells that had migrated through to the lower surface of the filter insert were stained with a 0.1% (w/v) solution of crystal violet (*Merck*) made up in 25% (v/v) methanol (*Merck*). A total of three random areas were chosen and a minimum of 1 cell and a maximum of 300 cells stained with crystal violet were counted under a Axio Scope 1 light microscope (*Zeiss*) at 400  $\times$  magnification. Migration rates were expressed as total percentage of the control. Data are representative of the three independent repeats and presented as mean  $\pm$  SEM.

#### Animal model

Prior to the commencement of the study all animal procedures were approved by both the animal care and ethics committee at Ulster University and the UK Home Office. Additionally, all animal procedures were carried out by licensed personnel in accordance with both local animal welfare committee (Ulster University) and national (UK Home Office) guidelines (Workman et al. 2010). For breeding purposes male *Apc*<sup>min/+</sup> mice were housed together with female wild type (*wt*) mice and all the animals subjected to a 12/12 light cycle, with food and water being available *ad libitum*. Mice were genotyped as described previously in Callaghan *et al.*, (2016), with both heterozygous *Apc*<sup>min/+</sup> and *wt* mice (male and female) used in experiments. During the study mice were monitored daily for grooming activity, general behaviour, activity levels, food and water intake and general wellbeing. Mice

assessed as exhibiting signs of distress or discomfort were immediately removed from the study and euthanized via CO<sub>2</sub> asphyxiation.

#### ASL dosing

At five weeks of age, both *wt* littermate and *Apc*<sup>min/+</sup> mice were treated via oral gavage every other day with either vehicle-only or a solution containing 50 mg kg<sup>-1</sup> (body weight) of ASL suspended in saline for 70 days. During treatment, body weights, general health and behaviour were monitored bi-weekly. Food and water were weighed on a weekly basis to determine the effect of ASL treatment on eating/drinking habits. A cut-off point for body weight reduction of 10% was applied and mice reaching this point were euthanized via CO<sub>2</sub> euthanasia.

#### Tissue collection and assessment

At the end of the experimental protocol, mice were euthanized with an overdose of pentobarbitone (200 mg kg<sup>-1</sup> given IP). A cardiac puncture was performed to collect blood that was stored in EDTA tubes (*Aquillant Scientific*). An additional blood sample was taken for haematocrit level estimation. For this a blood capillary tube was filled with blood, sealed, and centrifuged (Microcentrifuge with Hematocrit Rotor; *Cole-Parmer*) at 13,000 × *g* for 5 minutes. Haematocrit levels were calculated using the following calculation: height of RBC/total height of all the components × 100. Internal organs including the intestinal tract, colon, spleen, heart, liver, kidneys and lungs were removed, weighed and immerse fixed in 10% buffered formal saline at pH7.4 (*ThermoFisher Scientific*). The intestinal tracts were divided into 3 sections according to the description provided by Casteleyn *et al.*, (2010). After identification of the specific intestinal regions, samples were bisected longitudinally, and the total number of polyps was recorded as well as their diameters measured with callipers (Casteleyn *et al.* 2010). The specimens were then cut into ~2 cm strips and placed in cassettes prior to standard wax embedding. To assess qualitative histopathological changes in the intestines and spleen, tissues were cut into 5 µm sections using a Shandon Finesse 325 Microtome (*ThermoFisher Scientific*) placed on glass slides, cleared with xylene, dehydrated in descending grades of ethanol and subsequently stained with Mayer's haematoxylin and eosin (*Merck*). Stained sections were examined with a Axio Scope 1 light microscope (*Zeiss*) at a range of objective magnifications.

#### Statistical analyses

Statistical analysis of cell viability data was determined by two-way ANOVA followed by both Tukey's and Dunnett's post hoc testing. Analysis of live/ dead cell counts was determined by two-way ANOVA followed by Šidák's post hoc testing. Analysis of remaining *in vitro* data was determined by two-way ANOVA followed by both Dunnett's post hoc testing. Comparisons between *in vivo* groups was assessed using a students' t-test. A value of  $p \leq 0.05$  was considered statistically significant. All statistical analysis was carried out with the aid of Prism Version 9.3.1 (350) (GraphPad Software).

## Results

### Production and purification of ASL

A purified form of diacetylated ASL was produced for use in in this study from a commercially available SL formulation using methodologies outlined by Baccile *et al.* (2013). Following the recommendation of Twigg *et al.* 2021, the purified sample was analyzed via HPLC-ELSD and was found to be composed of 94% nonacetylated ASL. A breakdown of the various nonacetylated ASL congeners present in the samples can be seen in Table S1. The most abundant congener present in the sample was acidic C18:1s (75% relative abundance). The acidic C18:1t and C18:2t congeners represented 10% each, while all other ASL congeners were limited to less than 2% relative abundance (Twigg *et al.* 2021).

ASL have a selective effect on colorectal cancer cell viability.

Several colorectal tumour cell lines (HT29, HT115, HCT116 and Caco2), in addition to a colonic epithelial cell line (CCD-841-CoN) were treated with ASL to assess the effect on cell viability. ASL at concentrations up to 100  $\mu\text{g mL}^{-1}$  effected no significant decrease in the viability of the CCD-841-CoN cell line after 24 hours of treatment in comparison to cultures treated with a vehicle-only control (PBS) (Fig. 1a). However, 24 hours of treatment with ASL concentrations equal to or above 1  $\mu\text{g mL}^{-1}$  resulted in significantly reduced viability in the HT115 cell line in comparison to vehicle-only controls ( $p = 0.0201$ ) (Fig. 1a). In HT29, Caco2 and HCT116 cell lines, treatment with ASL concentrations equal to or above 10  $\mu\text{g mL}^{-1}$  resulted in a significant reduction in cell viability in comparison with cells treated with vehicle-only controls ( $p = < 0.0001$ ,  $< 0.0001$ , and 0.0099 respectively) (Fig.

1a). The viability of colorectal tumour cell lines HT29, HT115 and Caco2 were significantly reduced when compared to the colonic epithelial cell line CCD-841-CoN when treated with ASL concentrations equal to or above  $1 \mu\text{g mL}^{-1}$  ( $p = 0.0146$ ,  $< 0.0001$ , and  $0.0017$  respectively). For cell line HCT116 a significant reduction in viability when compared to CCD-841-CoN was observed when cells were treated with ASL concentrations equal to or above  $10 \mu\text{g mL}^{-1}$  ( $p = 0.0046$ ) (Fig. 1a).

ASL induces cell rounding, detachment and cell death in colorectal cancer cells

To observe potential effects on cell morphology, colorectal cancer cell lines (HT29, HT115, HCT116 and Caco2) and the colonic epithelial cell line (CCD-841-CoN) were treated with ASL and assessed by microscopic examination. CCD-841-CoN cells treated with a vehicle-only control (PBS) grew as a confluent monolayer with a bipolar morphology (Fig. S2). Following treatment with either  $20$  or  $70 \mu\text{g mL}^{-1}$  ASL no morphological differences of cells in the monolayer were observed when compared to vehicle-only treated CCD-841-CoN cells (Fig. S2). HT29, HT115, HCT116 and Caco2 treated with the vehicle-only control displayed a densely packed, cobblestone-like monolayer morphology (Fig. S2). When treated with ASL, disruption to the monolayer and detachment of HT-29, HT115, HCT116 and Caco-2 cell lines was observed. Partially detached and rounded cells were conspicuous within HT29, HT115 and Caco2 cell lines treated with  $20 \mu\text{g mL}^{-1}$  (Fig. S2). HT29, HT115, HCT116 and Caco2 cells exposed to  $70 \mu\text{g mL}^{-1}$  ASL, demonstrated extensive monolayer disruption with distinctive cell free areas within the cultures (Fig. S2).

As cellular detachment was observed in all tumour cell cultures, cells were isolated from the supernatant and stained with syto9 and propidium iodine to determine if detachment resulted in cell death. Only a small number of CD-841-CoN cells treated with either concentration of ASL were observed to detach and there was no significant difference in the proportion of these detached cells that were either alive or dead (Fig. 1b). In contrast the number of detached HT29, HT115, HCT116 and Caco2 cells was higher when treated with either concentration of ASL when compared to vehicle-only treated cells, and there was a significant increase in the proportion of these detached cells that were found to be dead (Fig. 1b).

ASL induces cell death *in vitro* via both apocopic and necrotic pathways.

As treatment with ASL resulted in a significant loss of cell viability, and induced detachment and cell death in colorectal cancer cell lines, the mechanism of cell death was investigated. Colorectal cancer (HT29, HT115 and HCT116 and Caco2) and colonic epithelial (CCD-841-CoN) cells lines were treated with ASL and stained with acridine orange and ethidium bromide to determine whether cell death was morphologically identifiable as a result of apoptosis or necrosis. Treatment of CCD-841-CoN cells with 20  $\mu\text{g mL}^{-1}$  of ASL did not result in a significant increase in the numbers of either apoptotic or necrotic cells. However, treatment with 70  $\mu\text{g mL}^{-1}$  ASL resulted in 10% cell death within the culture that was identified morphologically as apoptosis ( $p < 0.05$ ) (Fig. 1c). A dose-dependent increase in cell death in all four human colorectal cancer cell lines was observed following ASL treatment (Fig. 1C). In HT29, HT115 and HCT116 cell lines treated with 20  $\mu\text{g mL}^{-1}$  ASL, there were equivalent numbers of apoptotic and necrotic cells. However, there was a higher number of apoptotic cells in Caco2 cells treated with 20  $\mu\text{g mL}^{-1}$  ASL ( $p < 0.01$ ) (Fig. 1c). Exposure of cultures to 70  $\mu\text{g mL}^{-1}$  ASL, resulted in a markedly reduced number of adherent colorectal cancer cells available for quantification. HT29 and HT115 cultures treated with 70  $\mu\text{g mL}^{-1}$  showed a significant increase in the numbers of apoptotic and necrotic cells ( $p < 0.001$ ,  $p < 0.01$  respectively) while HCT116 cells were predominantly necrotic ( $p < 0.0001$ ), and Caco-2 cells had a higher number of apoptotic cells ( $p < 0.001$ ) compared to vehicle-only controls (Fig. 1c).

ASL reduces motility and anchorage-independent growth of tumour cells.

To assess the effect of ASL on cell migration, a scratch was made across a monolayer of colorectal cell lines HT29 and HCT115, and the colonic epithelial cell line CCD-841-CoN. Treatment with 10  $\mu\text{g mL}^{-1}$  ASL had no significant effect on CCD-841-CoN cell migration, with 90% of the total scratch area being covered after 72 hours (Fig. 2a). In contrast, treatment with 10  $\mu\text{g mL}^{-1}$  ASL resulted in a highly significant decrease in the proportion of the total scratch area covered in HT-29 (10%;  $p < 0.0001$ ) and HT115 cells (22%;  $p < 0.0001$ ) after 72 hours (Fig. 2a). To test the effect ASL had on chemotaxis, the same cell lines were plated in the upper portion of a Boyden chamber and the number of cells migrating in response to FCS were counted after 24 hours. Media supplemented with either 10 or 50  $\mu\text{g mL}^{-1}$  ASL did not significantly affect the migration of CCD-841-CoN cells. The addition of 10  $\mu\text{g mL}^{-1}$  ASL to media did however result in a significant reduction in migration of HT29 (33%) and HT115 (29%) compared to control values ( $p < 0.001$ ) (Fig. 2b). The addition of 50  $\mu\text{g mL}^{-1}$  ASL reduced migration of HT29 and HT115 to 8.3% and 10.4% of control values respectively ( $p < 0.0001$ ) (Fig. 2b).

*In vivo* experiments: *wt* and *Apc*<sup>min/+</sup> mice tolerate the oral administration of ASL

To determine palatability and potential toxicity of ASL, a pilot study was performed with both *wt* and *Apc*<sup>min/+</sup> mice which were fed either a vehicle-only control solution consisting of 10% sucrose water or vehicle containing either 0.5, 5 or 50 mg kg<sup>-1</sup> ASL for a period of 5 weeks (n = 3/group). Both *wt* and *Apc*<sup>min/+</sup> mice gained weight at a similar rate and there was no change in food or water intake between any of the treatment groups (Fig. S3). On completion of this oral feeding tolerance study, the major organs (liver, stomach, kidneys, lungs, heart, spleen and pancreas) were removed, weighed and a gross inspection of morphology was carried out. There were no significant differences in organ weights (Table S2) or gross organ morphology observed between mice fed on the vehicle only control diet or on ASL.

ASL reduces polyp-associated bleeding and increases haematocrit levels but does not affect tumour size or numbers in *Apc*<sup>min/+</sup> mice.

The gross morphological appearances of unfixed, flat-mounted ilea from *wt* mice treated with either vehicle-only control or 50 mg kg<sup>-1</sup> ASL was characterised by a flattened, uniformly smooth mucous epithelium with prominent patent blood vessels (Fig. 3a). In vehicle-only treated *Apc*<sup>min/+</sup> mice, there was clear evidence of polyp-associated bleeding within the ileal segment (Fig. 3a). In contrast, *Apc*<sup>min/+</sup> mice treated with 50 mg kg<sup>-1</sup> ASL for 70 days, showed little evidence of bleeding from these intestinal polyps (Fig. 3a). However, the number of intestinal polyps in *Apc*<sup>min/+</sup> mice was not significantly different following treatment with 50 mg kg<sup>-1</sup> ASL for 70 days in comparison the vehicle-only control treated cohort (vehicle = 48 ± 2 vs ASL = 45 ± 4; *p* < 0.1) (Fig. 3b). ASL treatments also had no effect on the modal size distribution of the polyps in comparison to vehicle-only treatment (vehicle- 4 mm vs ASL 4 mm; *p* > 0.05) (Fig. 3c).

No significant difference in hematocrit level were observed in *wt* mice fed with either vehicle-only control or 50 mg kg<sup>-1</sup> ASL for 35 or 70 days (*p* = 0.8132 and *p* = 0.9685 respectively) (Fig. 3d and Fig. 3e). Hematocrit levels in *wt* mice were also significantly higher than those of *Apc*<sup>min/+</sup> mice, irrespective of treatment with either vehicle-only control or ASL (*p* = > 0.05) (Fig. 3d and Fig. 3e). After 35 days feeding with 50 mg kg<sup>-1</sup> ASL, no significant differences in haematocrit levels were observed in *Apc*<sup>min/+</sup> mice compared to those fed with vehicle-only control (vehicle-only = 41.2 ± 0.8 vs ASL = 40.0 ± 1.1; *p* = 0.9906) (Fig. 3d). However, following 70 days of administration with 50 mg kg<sup>-1</sup> ASL, haematocrit levels were found to be significantly higher in

Apc<sup>min/+</sup> mice fed in comparison to those fed with the vehicle-only control (vehicle-only = 36.0 ± 0.9 vs ASL = 42 ± 1.9; *p* = 0.012) (Fig. 3e).

ASL effects the splenic weight, proportion of spleen red pulp in Apc<sup>min/+</sup> mice

The spleens from vehicle-only control fed Apc<sup>min/+</sup> mice were significantly heavier than their *wt* littermates (0.58 ± 0.2g vs 0.15g ± 0.5g; *p* < 0.0001). A dose of 50 mg kg<sup>-1</sup> ASL had no effect on splenic weight in *wt* mice after 70 days of treatment. However, administration of 50 mg kg<sup>-1</sup> ASL for 70 days to Apc<sup>min/+</sup> mice resulted in a statistically significant decrease in splenic weight (0.58 ± 0.2 g vs 0.40 ± 0.4g; *p* < 0.001) (Fig. 4a). Following treatment of the Apc<sup>min/+</sup> mice with 50 mg kg<sup>-1</sup> ASL there was a significant reduction in the proportion of red pulp as compared with vehicle-only controls (62 ± 3.2 vs 48 ± 1.4; *p* < 0.001), no significant difference was observed in the *wt* mice (Fig. 4b). Histological examination of sections from *wt* mouse spleen revealed conspicuous and intensely basophilic areas of white pulp; these were separated by less dense regions of red pulp; areas that are responsible for removal of old or damaged erythrocytes. In vehicle-only control treated Apc<sup>min/+</sup> mice there was obvious clumping of the red pulp, and the proportion of this tissue was significantly increased when compared to *wt* mice (Fig. 4c). Apc<sup>min/+</sup> mice fed with ASL showed reduced clumping of red pulp and an increase in the white pulp regions, like that of the *wt* mice (Fig. 4c).

## Discussion

The ability of chemotherapeutic agents to selectively target cancerous cells while sparing normal tissue is a highly desirable trait, as it may mitigate against the common side effects associated with these toxic therapeutics such as epithelial cell damage in the gastrointestinal tract, immunosuppression (in the bone marrow) and hair loss (Carey and Burish 1988; McQuade et al. 2014). Biosurfactant compounds such as sophorolipids are naturally produced agents that may possess the ability to differentially affect cancer and normal epithelial cells and as such their anti-cancer activities are a growing area of research. Here we investigated the *in vitro* effects of a 94% pure preparation of ASL on a non-transformed intestinal epithelial cell line (CCD-841-CoN) and 4 colorectal cancer cell lines (HT29, HT115, Caco2, and HCT116). Colonic epithelia (CCD-841-CoN) treated with ASL showed no sign of toxicity as evidenced by maintenance of their viability at doses ranging between 0.001 and 100 µg mL<sup>-1</sup>. However, at doses of 20 µg mL<sup>-1</sup> and above, ASL's potently reduced cell viability in all the colorectal cancer cell lines



examined. Although the cytotoxic potency of sophorolipid mixtures has been reported against cancer cell lines previously, (Chen et al. 2006b; Fu et al. 2008; Shao et al. 2012; Ribeiro et al. 2015; Callaghan et al. 2016; Adu et al. 2022), this is the first time an anti-cancer effect has been reported from a purified and well characterised ASL on a range of cell lines from the same tissue of origin (i.e. colorectal cancer cells).

Importantly, the ASL preparation utilised here exhibited a differential effect *in vitro* on non-transformed cell lines compared to CRC cells, with a dose-dependent cytotoxic response in all five CRC cell lines. A differential effect has been reported in previous studies where SL were cytotoxic to pancreatic, liver and melanoma cancer cell lines but with no demonstrated toxicity to non-transformed cell lines (Fu et al. 2008; Adu et al. 2022). However, Fu *et al.* 2008 utilised non-adherent circulating blood monocytes (PBMC) as a control for adherent transformed cells making conclusions from this study difficult to interpret. Therefore, other than Adu and colleagues work on melanoma cell lines, a directly comparable description of the specificity of a pure preparation of ASL that uses a normal adherent cell line from the same tissue of origin has not been carried out.

It has been hypothesised that SL can intercalate into the cytoskeleton of cells resulting in membrane disruption. Changes in cell morphology consistent with disruption of membrane cytoskeletal protein distribution have previously been shown to occur with SL doses as low as 30  $\mu\text{g mL}^{-1}$  in pancreatic H7402 and lung cancer A549 cell lines (Chen et al. 2006b). Our study demonstrated that a preparation dominated by a single congener of ASL at low concentrations (40  $\mu\text{g mL}^{-1}$ ) induced cell rounding, cytoplasmic condensation and cell detachment in all the CRC cancer cell lines tested, with the adenocarcinoma cell lines HT29 and Caco2 being most susceptible. At doses of 70 – 100  $\mu\text{g mL}^{-1}$ , ASL caused a significant increase in the numbers of detached cells with all cells in the supernatant from treated cultures showing morphological features consistent with apoptosis or necrosis. Similar results have been observed in HepG2 liver cancer cells treated with LSL preparations (Wang et al. 2021).

ASL's used at the doses described in our study, induced a reduction of tension at the interfacial region of the bilayer resulting in phospholipid dehydration which affected lipid stability and ultimately resulted in cell death (Maget-Dana et al. 1989; Shah et al. 2005; Ortiz et al. 2009). To assess cell death mechanisms caused by ASL's, ethidium bromide/acridine orange staining was carried out allowing the morphological identification of the type of cell death. We have conclusively demonstrated that ASL induces both apoptosis and necrosis in these cancer cell lines in a dose-dependent manner. The ability of ASL to induce either apoptosis or necrosis may be cell-line specific, as high proportions of apoptotic cells have been reported following the addition of diacetylated LSL to liver (H7402) and lung cancer (H7402) cell cultures, while necrosis is primarily observed in a pancreatic

carcinoma cell line (HPAC) treated with sophorolipid mixtures, LSL or methyl ester derivative SL (Shao et al. 2012).

An important characteristic of malignant growth is the ability of tumour cells to leave their restricted compartment and gain access to blood vessels to initiate the first phase of metastasis (Hanahan and Weinberg 2011). The movement of cells across tissues therefore plays an important role in this progression thus highlighting the need for an agent that can counteract the migratory and diapedesis properties of colorectal cancer cells (Dianzani et al. 2014). It has been postulated that the amphiphilic properties of sophorolipids permit their incorporation into the mammalian cellular membrane disrupting cellular functions such as proliferation and migration (Zhao et al. 2013; Haque et al. 2021; Adu et al. 2022). We assessed the ability of ASL's to inhibit migration of the CRC cell lines HT29 and HT115. At a low dose ( $10 \mu\text{g mL}^{-1}$ ) of ASL there was no effect on normal colonic epithelial cells (CCD-841-CoN) and scratch coverage after 72 hours. However, the same dose of ASL applied to colorectal cancer cell lines HT29 and HT115 cultures reduced the total percentage area of scratch covered to between 12-25%. An inhibition of migration induced by sophorolipid (as measured in the scratch assay) has been documented. In 2015, Riberiro and co-workers showed that  $5 \mu\text{g mL}^{-1}$  of a 93% pure, diacetylated LSL resulted in significantly reduced migration of MDA-MB-231 breast cancer cells (Ribeiro et al. 2015), although to our knowledge no reports are available for a study that solely utilises purified ASL. Adu *et al.* 2022 showed that the migration of a melanoma cell line SK-MEL-28 was significantly reduced when treated with LSL and ASL preparations in comparison with cells treated with a vehicle-only control, and immortalized keratinocytes (HaCaT) treated with the same sophorolipid preparations (Adu et al. 2022). The use of both migration and invasion assays incorporating the Boyden chamber are widely used to test candidate chemotherapeutics with the HT29 and HT115 cell lines commonly employed to test anti-invasive properties of potential chemotherapeutics *in vitro* (Li and Zhu 1999). Currently the only studies investigating the potential anti-metastatic properties of biosurfactants are limited to the inhibitory effects on breast cancer cell lines MCF and MDA-MB-231 *in vitro* invasion mediated by the lipopeptide Surfactin produced by *Bacillus subtilis* (Park et al. 2013). Addition of  $10 \mu\text{M}$  (equivalent to approx.  $660 \mu\text{g mL}^{-1}$  of ASL in this study) of Surfactin had the ability to reduce the migration of MCF-7 and MDA-MB-231 through an extracellular matrix by 68% and 84% respectively (Park et al. 2013). The Surfactin used by Park *et al.* (2013) also reduced the colony forming ability of both cell lines by 70% and 61%. To investigate the migration inhibitory properties of ASL, HT29 and HT115 cells were plated on a porous membrane and allowed to migrate through a septum in response to a FBS stimulus in the chamber below. ASL had no effect on the migration of normal colonic CCD-841-CoN cells, however following treatment with  $10 \mu\text{g mL}^{-1}$  and  $50 \mu\text{g mL}^{-1}$

ASL, migration was significantly decreased in HT29 cell lines by 65% and 72% respectively, and 86% and 81% in HT115 cell lines.

The anti-tumour activities of sophorolipid preparations *in vivo* are unclear. In a study by Li and co-workers, an intragastric administered mixture composed of 6 di-acetylated LSL congeners was shown to reduce the size of tumours in a murine HeLa xenograft model (Li et al. 2017). Whereas a purified form of LSL exaggerated the growth of neoplasm along the intestinal tract and increased intestinal blood loss in the  $Apc^{min/+}$  mouse (Callaghan et al. 2016). In this study, we use the  $Apc^{min/+}$  mouse model of FAP, to evaluate the chemotherapeutic potential of purified ASL in the treatment of solid neoplasms.  $Apc^{min/+}$  mice develop intestinal adenomatous neoplasms (polyps), and animals typically present with enlarged spleens and reduced haematocrit by 4 months of age (Yekkala and Baudino 2007). This is an acute model with a life span of <150 days, where the primary cause of death is not directly attributable to the development of numerous polyps but rather as a result of extensive intestinal bleeding and anaemia (Hinoi et al. 2007). When administered orally, topically or via *i.v.* injection sophorolipid mixtures are well tolerated (Ikeda et al. 1986). Similarly, in our study, both *wt* and  $Apc^{min/+}$  mice tolerated the oral administration of ASL with no measurable gross anatomical or behavioural differences noted. Post-mortem analysis also revealed no effects on gross measures of peritoneal organs (size and dimensions) after ASL treatment. However, oral feeding of ASL to  $Apc^{min/+}$  for 70 days resulted in a reduction of spleen size and a significant increase in haematocrit, consistent with decreased intestinal bleeding and improvement in the associated anaemia characteristic of this model (Perkins et al. 2002; Hinoi et al. 2007). This is a potentially significant finding, as rectal bleeding and anaemia are reported in over 30% of CRC patients and it is a contributing factor in reduced lifespan in both humans as well as  $Apc^{min/+}$  mice (Ronnekleiv-Kelly and Kennedy 2011). In humans, laser ablation encourages coagulation of tumours demonstrating significant blood loss and this technique has shown to be effective after 2 - 5 treatments with a success rate of 80% (Kimmey 2004). However recurrent bleeding episodes result in surgical intervention in 2 - 15% of patients (Rao et al. 2005). The oral administration of a well-tolerated non-toxic, pro-thrombotic agents to reduce intestinal blood loss in patients with haemorrhagic colorectal tumours may be a useful addition to the therapeutic treatment of these conditions.

In conclusion, the purified ASL mixture we utilised in this study differentially affects non-transformed in comparison to colorectal cancer cell lines, resulting in a significant and dose-dependent decrease in their viability, migration, and anchorage-independent growth characteristics. While ASL does not change either the size or number of intestinal polyps in  $Apc^{min/+}$  mice, both spleen size and tumour-associated bleeding was reduced.

This warrants further investigation of this orally available biosurfactant as a chemotherapeutic for delaying disease progression in pre-cancerous colorectal neoplasms.

## Author Contributions

BC and MST contributed equally to this work. INAVB, RM, CM and IMB conceived and designed the research. BC and NB conducted experiments. BC and MST analysed data and wrote the manuscript. All authors read and approved the manuscript.

## References

- Adu SA, Naughton PJ, Marchant R, Banat IM (2020) Microbial Biosurfactants in Cosmetic and Personal Skincare Pharmaceutical Formulations. *Pharmaceutics* 12:1099
- Adu SA, Twigg MS, Naughton PJ, Marchant R, Banat IM (2022) Biosurfactants as Anticancer Agents: Glycolipids Affect Skin Cells in a Differential Manner Dependent on Chemical Structure. *Pharm.* 2022, Vol. 14, Page 360 14:360
- Altman SA, Randers L, Rao G (1993) Comparison of Trypan Blue Dye Exclusion and Fluorometric Assays for Mammalian Cell Viability Determinations. *Biotechnol Prog* 9:671–674. <https://doi.org/10.1021/bp00024a017>
- Baccile N, Cuvier AS, Prévost S, Stevens C V., Delbeke E, Berton J, Soetaert W, Van Bogaert INA, Roelants S (2016) Self-Assembly Mechanism of pH-Responsive Glycolipids: Micelles, Fibers, Vesicles, and Bilayers. *Langmuir* 32:10881–10894. <https://doi.org/10.1021/acs.langmuir.6b02337>
- Baccile N, Cuvier AS, Valotteau C, Van Bogaert INA (2013) Practical methods to reduce impurities for gram-scale amounts of acidic sophorolipid biosurfactants. *Eur J Lipid Sci Technol* 115:1404–1412. <https://doi.org/10.1002/ejlt.201300131>
- Banat IM, Franzetti A, Gandolfi I, Bestetti G, Martinotti MG, Fracchia L, Smyth TJ, Marchant R (2010) Microbial biosurfactants production, applications and future potential. *Appl Microbiol Biotechnol* 87:427–444.

562 <https://doi.org/10.1007/s00253-010-2589-0>  
 563 Barbuti AM, Chen ZS (2015) Paclitaxel through the ages of anticancer therapy: Exploring its role in  
 564 chemoresistance and radiation therapy. *Cancers (Basel)*. 7:2360–2371  
 565 Beutler JA (2019) Natural Products as a Foundation for Drug Discovery. *Curr. Protoc. Pharmacol.* 86:e67  
 566 Bluth M, Fu S, Fu A, Stanek A, Smithnorowitz T, Wallner S, Gross R, Nowakowski M, Zenilman M (2008)  
 567 Sophorolipids Decrease Asthma Severity And Ova-specific IgE Production In A Mouse Asthma Model. *J*  
 568 *Allergy Clin Immunol* 121:S2–S2. <https://doi.org/10.1016/j.jaci.2007.12.011>  
 569 Callaghan B, Lydon H, Roelants SLKW, Van Bogaert INA, Marchant R, Banat IM, Mitchell CA (2016) Lactonic  
 570 Sophorolipids Increase Tumor Burden in Apc<sup>min+/-</sup> Mice. *PLoS One* 11:e0156845  
 571 Carey MP, Burish TG (1988) Etiology and Treatment of the Psychological Side Effects Associated With Cancer  
 572 Chemotherapy: A Critical Review and Discussion. *Psychol Bull* 104:307–325.  
 573 <https://doi.org/10.1037/0033-2909.104.3.307>  
 574 Casas J, García-Ochoa F (1999) Sophorolipid production by *Candida bombicola*: Medium composition and  
 575 culture methods. *J Biosci Bioeng* 88:488–494. [https://doi.org/https://doi.org/10.1016/S1389-](https://doi.org/https://doi.org/10.1016/S1389-1723(00)87664-1)  
 576 [1723\(00\)87664-1](https://doi.org/https://doi.org/10.1016/S1389-1723(00)87664-1)  
 577 Casteleyn C, Doom M, Lambrechts E, van den Broeck W, Simoens P, Cornillie P (2010) Locations of gut-  
 578 associated lymphoid tissue in the 3-month-old chicken: A review. *Avian Pathol* 39:143–150.  
 579 <https://doi.org/10.1080/03079451003786105>  
 580 Ceresa C, Fracchia L, Fedeli E, Porta C, Banat IM (2021) Recent advances in biomedical, therapeutic and  
 581 pharmaceutical applications of microbial surfactants. *Pharmaceutics* 13:466.  
 582 <https://doi.org/10.3390/pharmaceutics13040466>  
 583 Chen HC (2005) Boyden chamber assay. In: Guan J-L (ed) *Methods in molecular biology* (Clifton, N.J.). Humana  
 584 Press, Totowa, NJ, pp 15–22  
 585 Chen J, Song X, Zhang H, Qu Y (2006a) Production, structure elucidation and anticancer properties of  
 586 sophorolipid from *Wickerhamiella domercqiae*. *Enzyme Microb Technol* 39:501–506.  
 587 <https://doi.org/https://doi.org/10.1016/j.enzmictec.2005.12.022>  
 588 Chen J, Song X, Zhang H, Qu YB, Miao JY (2006b) Sophorolipid produced from the new yeast strain  
 589 *Wickerhamiella domercqiae* induces apoptosis in H7402 human liver cancer cells. *Appl Microbiol*  
 590 *Biotechnol* 72:52–59. <https://doi.org/10.1007/s00253-005-0243-z>  
 591 D’Incalci M, Steward WP, Gescher AJ (2005) Use of cancer chemopreventive phytochemicals as antineoplastic

agents. *Lancet Oncol* 6:899–904. [https://doi.org/10.1016/s1470-2045\(05\)70425-3](https://doi.org/10.1016/s1470-2045(05)70425-3)

Dhar S, Reddy EM, Prabhune A, Pokharkar V, Shiras A, Prasad BL V (2011) Cytotoxicity of sophorolipid-gellan gum-gold nanoparticle conjugates and their doxorubicin loaded derivatives towards human glioma and human glioma stem cell lines. *Nanoscale* 3:575–580. <https://doi.org/10.1039/C0NR00598C>

Dianzani C, Minelli R, Gigliotti CL, Occhipinti S, Giovarelli M, Conti L, Boggio E, Shivakumar Y, Baldanzi G, Malacarne V, Orilieri E, Cappellano G, Fantozzi R, Sblattero D, Yagi J, Rojo JM, Chiocchetti A, Dianzani U (2014) B7h Triggering Inhibits the Migration of Tumor Cell Lines. *J Immunol* 192:4921–4931. <https://doi.org/10.4049/jimmunol.1300587>

Elshikh M, Funston S, Chebbi A, Ahmed S, Marchant R, Banat IM (2017) Rhamnolipids from non-pathogenic *Burkholderia thailandensis* E264: Physicochemical characterization, antimicrobial and antibiofilm efficacy against oral hygiene related pathogens. *N Biotechnol* 36:26–36. <https://doi.org/10.1016/j.nbt.2016.12.009>

Ferlay J, Ervik M, Lam F, Colombet M, Mery L, Piñeros M, Znaor A, Soerjomataram I, Bray F (2020) Global Cancer Observatory: Cancer Today. <https://gco.iarc.fr/today>. Accessed 3 Dec 2021

Flam F (1994) Race to synthesize taxol ends in a tie. *Science* (80-. ). 263:911

Fridlender M, Kapulnik Y, Koltai H (2015) Plant derived substances with anti-cancer activity: From folklore to practice. *Front Plant Sci* 6:799. <https://doi.org/10.3389/fpls.2015.00799>

Fu SL, Wallner SR, Bowne WB, Hagler MD, Zenilman ME, Gross R, Bluth MH (2008) Sophorolipids and Their Derivatives Are Lethal Against Human Pancreatic Cancer Cells. *J Surg Res* 148:77. <https://doi.org/10.1016/j.jss.2008.03.005>

Hanahan D, Weinberg RA (2011) Hallmarks of cancer: The next generation. *Cell* 144:646–674

Haque F, Khan MSA, AlQurashi N (2021) ROS-Mediated Necrosis by Glycolipid Biosurfactants on Lung, Breast, and Skin Melanoma Cells. *Front. Oncol.* 11:253

Hardin R, Pierre J, Schulze R, Mueller CM, Fu SL, Wallner SR, Stanek A, Shah V, Gross RA, Weedon J, Nowakowski M, Zenilman ME, Bluth MH (2007) Sophorolipids Improve Sepsis Survival: Effects of Dosing and Derivatives. *J Surg Res* 142:314–319. <https://doi.org/10.1016/j.jss.2007.04.025>

Hinoi T, Akyol A, Theisen BK, Ferguson DO, Greenson JK, Williams BO, Cho KR, Fearon ER (2007) Mouse model of colonic adenoma-carcinoma progression based on somatic Apc inactivation. *Cancer Res* 67:9721–9730. <https://doi.org/10.1158/0008-5472.CAN-07-2735>

Ikeda Y, Sunakawa T, Tsuchiya S, Kondo M, Okamoto K (1986) Toxicological studies on sophorolipid derivatives. (I) Acute toxicity, eye irritation, primary skin irritation, skin sensitization, phototoxicity,

622 photosensitization, mutagenicity of polyoxypropylene (12) [(2'-0- $\beta$ -D-glucopyranosyl- $\beta$ -D-  
 623 glucopyranosyl)oxy-]. J Toxicol Sci 11:197–211. <https://doi.org/10.2131/jts.11.197>  
 624 Kelloff GJ, Boone CW, Crowell JA, Steele VE, Lubet R, Sigman CC (1994) Chemopreventive drug development:  
 625 perspectives and progress. Cancer Epidemiol biomarkers Prev a Publ Am Assoc Cancer Res cosponsored  
 626 by Am Soc Prev Oncol 3:85–98  
 627 Khalifa SAM, Elias N, Farag MA, Chen L, Saeed A, Hegazy MEF, Moustafa MS, El-Wahed AA, Al-Mousawi  
 628 SM, Musharraf SG, Chang FR, Iwasaki A, Suenaga K, Alajlani M, Göransson U, El-Seedi HR (2019)  
 629 Marine natural products: A source of novel anticancer drugs. Mar Drugs 17:491.  
 630 <https://doi.org/10.3390/md17090491>  
 631 Kimmey MB (2004) Endoscopic methods (other than stents) for palliation of rectal carcinoma. J Gastrointest Surg  
 632 8:270–273. <https://doi.org/10.1016/j.gassur.2003.11.017>  
 633 Li H, Guo W, Ma X, Li J, Song X (2017) *In Vitro* and *In Vivo* Anticancer Activity of Sophorolipids to Human  
 634 Cervical Cancer. Appl Biochem Biotechnol 181:1372–1387. <https://doi.org/10.1007/s12010-016-2290-6>  
 635 Li YH, Zhu C (1999) A modified Boyden chamber assay for tumor cell transendothelial migration *in vitro*. Clin  
 636 Exp Metastasis 17:423–429. <https://doi.org/10.1023/A:1006614232388>  
 637 Maget-Dana R, Harnois I, Ptak M (1989) Interactions of the lipopeptide antifungal iturin A with lipids in mixed  
 638 monolayers. BBA - Biomembr 981:309–314. [https://doi.org/10.1016/0005-2736\(89\)90042-4](https://doi.org/10.1016/0005-2736(89)90042-4)  
 639 Marchant R, Banat IM (2012a) Biosurfactants: A sustainable replacement for chemical surfactants? Biotechnol  
 640 Lett 34:1597–1605. <https://doi.org/10.1007/s10529-012-0956-x>  
 641 Marchant R, Banat IM (2012b) Microbial biosurfactants: Challenges and opportunities for future exploitation.  
 642 Trends Biotechnol. 30:558–565  
 643 Mazevet M, Moulin M, Llach-Martinez A, Chargari C, Deutsch É, Gomez A-M, Morel É (2013) Complications  
 644 of chemotherapy, a basic science update. Presse Med 42:e352–e361.  
 645 <https://doi.org/https://doi.org/10.1016/j.lpm.2013.06.011>  
 646 McQuade RM, Bornstein JC, Nurgali K (2014) Anti-Colorectal Cancer Chemotherapy-Induced Diarrhoea:  
 647 Current Treatments and Side-Effects. Int J Clin Med 05:393–406. <https://doi.org/10.4236/ijcm.2014.57054>  
 648 Morgan MA, Rubin SC (1998) Long-term complications of chemotherapy. Prim Care Update Ob Gyns 5:65–68.  
 649 [https://doi.org/https://doi.org/10.1016/S1068-607X\(98\)00010-9](https://doi.org/https://doi.org/10.1016/S1068-607X(98)00010-9)  
 650 Moser AR, Luongo C, Gould KA, McNeley MK, Shoemaker AR, Dove WF (1995) ApcMin: A mouse model for  
 651 intestinal and mammary tumorigenesis. Eur J Cancer 31:1061–1064. <https://doi.org/10.1016/0959->

8049(95)00181-H

- Naughton PJ, Marchant R, Naughton V, Banat IM (2019) Microbial biosurfactants: current trends and applications in agricultural and biomedical industries. *J Appl Microbiol* 127:12–28. <https://doi.org/10.1111/jam.14243>
- O’Connell MJ, Lavery I, Yothers G, Paik S, Clark-Langone KM, Lopatin M, Watson D, Baehner FL, Shak S, Baker J, Cowens JW, Wolmark N (2010) Relationship Between Tumor Gene Expression and Recurrence in Four Independent Studies of Patients With Stage II/III Colon Cancer Treated With Surgery Alone or Surgery Plus Adjuvant Fluorouracil Plus Leucovorin. *J Clin Oncol* 28:3937–3944. <https://doi.org/10.1200/JCO.2010.28.9538>
- Ortiz A, Teruel JA, Espuny MJ, Marqués A, Manresa Á, Aranda FJ (2009) Interactions of a bacterial biosurfactant trehalose lipid with phosphatidylserine membranes. *Chem Phys Lipids* 158:46–53. <https://doi.org/10.1016/j.chemphyslip.2008.11.001>
- Park SY, Kim JH, Lee YJ, Lee SJ, Kim Y (2013) Surfactin suppresses TPA-induced breast cancer cell invasion through the inhibition of MMP-9 expression. *Int J Oncol* 42:287–296. <https://doi.org/10.3892/ijo.2012.1695>
- Perkins S, Verschoyle RD, Hill K, Sharma RA, Williams ML, Steward WP, Gescher AJ, Parveen I, Threadgill MD (2002) Chemopreventive efficacy and pharmacokinetics of curcumin in the min/+ mouse, a model of familial adenomatous polyposis. *Cancer Epidemiol Biomarkers Prev* 11:535–540
- Rao VSR, Al-Mukhtar A, Rayan F, Stojkovic S, Moore PJ, Ahmad SM (2005) Endoscopic laser ablation of advanced rectal carcinoma - A DGH experience. *Color. Dis.* 7:58–60
- Reuter BK, Zhang X-J, Miller MJS (2002) Therapeutic utility of aspirin in the Apc<sup>Min/+</sup> murine model of colon carcinogenesis. *BMC Cancer* 2:19. <https://doi.org/10.1186/1471-2407-2-19>
- Ribeiro IAC, Faustino CMC, Guerreiro PS, Frade RFM, Bronze MR, Castro MF, Ribeiro MHL (2015) Development of novel sophorolipids with improved cytotoxic activity toward MDA-MB-231 breast cancer cells. *J Mol Recognit* 28:155. <https://doi.org/10.1002/jmr.2403>
- Roelants SLKW, Ciesielska K, De Maeseneire SL, Moens H, Everaert B, Verweire S, Denon Q, Vanlerberghe B, Van Bogaert INA, Van der Meeren P, Devreese B, Soetaert W (2016) Towards the industrialization of new biosurfactants: Biotechnological opportunities for the lactone esterase gene from *Starmerella bombicola*. *Biotechnol Bioeng* 113:550–559. <https://doi.org/10.1002/bit.25815>
- Roelants SLKW, Solaiman DKY, Van Renterghem L, Lodens S, Soetaert W, Ashby R (2019) Production and application of sophorolipids. In: Hayes DG, Ashby RD, Solaiman D. (eds) *Biobased Surfactants: Synthesis, Properties and Applications*, 2nd edn. AOCS Press, London, pp 65–119



682 Ronnekleiv-Kelly SM, Kennedy GD (2011) Management of stage IV rectal cancer: Palliative options. *World J*  
683 *Gastroenterol* 17:835–847. <https://doi.org/10.3748/wjg.v17.i7.835>

684 Sarubbo LA, Silva M da GC, Durval IJB, Bezerra KGO, Ribeiro BG, Silva IA, Twigg MS, Banat IM (2022)  
685 Biosurfactants: Production, Properties, Applications, Trends, and General Perspectives. *Biochem Eng J*  
686 108377. <https://doi.org/10.1016/j.bej.2022.108377>

687 Schneider CA, Rasband WS, Eliceiri KW (2012) NIH Image to ImageJ: 25 years of image analysis. *Nat Methods*  
688 9:671–675. <https://doi.org/10.1038/nmeth.2089>

689 Shah V, Doncel GF, Seyoum T, Eaton KM, Zalenskaya I, Hagver R, Azim A, Gross R (2005) Sophorolipids,  
690 microbial glycolipids with anti-human immunodeficiency virus and sperm-immobilizing activities.  
691 *Antimicrob Agents Chemother* 49:4093–4100. <https://doi.org/10.1128/AAC.49.10.4093-4100.2005>

692 Shao L, Song X, Ma X, Li H, Qu Y (2012) Bioactivities of Sophorolipid with Different Structures Against Human  
693 Esophageal Cancer Cells. *J Surg Res* 173:286–291. <https://doi.org/https://doi.org/10.1016/j.jss.2010.09.013>

694 Smyth, Thomas J. P, Perfumo, A., Marchant, R., Bannat, I.M. (2010) Isolation and anlysis of low molecular  
695 weight microbial glycolipids. In: *Handbook of Hydrocarbon and Lipid Microbiology*. Berlin Heidelberg,  
696 pp 3705–3723

697 Stierle A, Strobel G, Stierle D (1993) Taxol and taxane production by *Taxomyces andreanae*, an endophytic  
698 fungus of Pacific yew. *Science* (80- ) 260:214–216. <https://doi.org/10.1126/science.8097061>

699 Tucker JM, Davis C, Kitchens ME, Bunni MA, Priest DG, Spencer HT, Berger FG (2002) Response to 5-  
700 fluorouracil chemotherapy is modified by dietary folic acid deficiency in *Apc<sup>Min/+</sup>* mice. *Cancer Lett*  
701 187:153–162. [https://doi.org/10.1016/S0304-3835\(02\)00402-0](https://doi.org/10.1016/S0304-3835(02)00402-0)

702 Twigg MS, Baccile N, Banat IM, Déziel E, Marchant R, Roelants S, Van Bogaert INA (2021) Microbial  
703 biosurfactant research: time to improve the rigour in the reporting of synthesis, functional characterization  
704 and process development. *Microb. Biotechnol* 14:147-170. <https://doi.org/10.1111/1751-7915.13704>

705 Van Bogaert INA, Saerens K, De Muynck C, Develter D, Soetaert W, Vandamme EJ (2007) Microbial production  
706 and application of sophorolipids. *Appl Microbiol Biotechnol* 76:23–34. [https://doi.org/10.1007/s00253-](https://doi.org/10.1007/s00253-007-0988-7)  
707 007-0988-7

708 Van Bogaert INA, Zhang J, Soetaert W (2011) Microbial synthesis of sophorolipids. *Process Biochem.* 46:821–  
709 833

710 Wang X, Xu N, Li Q, Chen S, Cheng H, Yang M, Jiang T, Chu J, Ma X, Yin D (2021) Lactonic sophorolipid–  
711 induced apoptosis in human HepG2 cells through the Caspase-3 pathway. *Appl Microbiol Biotechnol*

105:2033–2042. <https://doi.org/10.1007/s00253-020-11045-5>

Workman P, Aboagye EO, Balkwill F, Balmain A, Bruder G, Chaplin DJ, Double JA, Everitt J, Farningham DAH, Glennie MJ, Kelland LR, Robinson V, Stratford IJ, Tozer GM, Watson S, Wedge SR, Eccles SA, Navaratnam V, Ryder S (2010) Guidelines for the welfare and use of animals in cancer research. *Br. J. Cancer* 102:1555–1577

Yekkala K, Baudino TA (2007) Inhibition of intestinal polyposis with reduced angiogenesis in *Apc<sup>Min/+</sup>* mice due to decreases in c-Myc expression. *Mol Cancer Res* 5:1296–1303. <https://doi.org/10.1158/1541-7786.MCR-07-0232>

Zhao J, Wu Y, Alfred AT, Xin X, Yang S (2013) Chemical structures and biological activities of rhamnolipid biosurfactants produced by *Pseudomonas aeruginosa* M14808. *J Chem Pharm Res* 5:177

## Fig. Legends

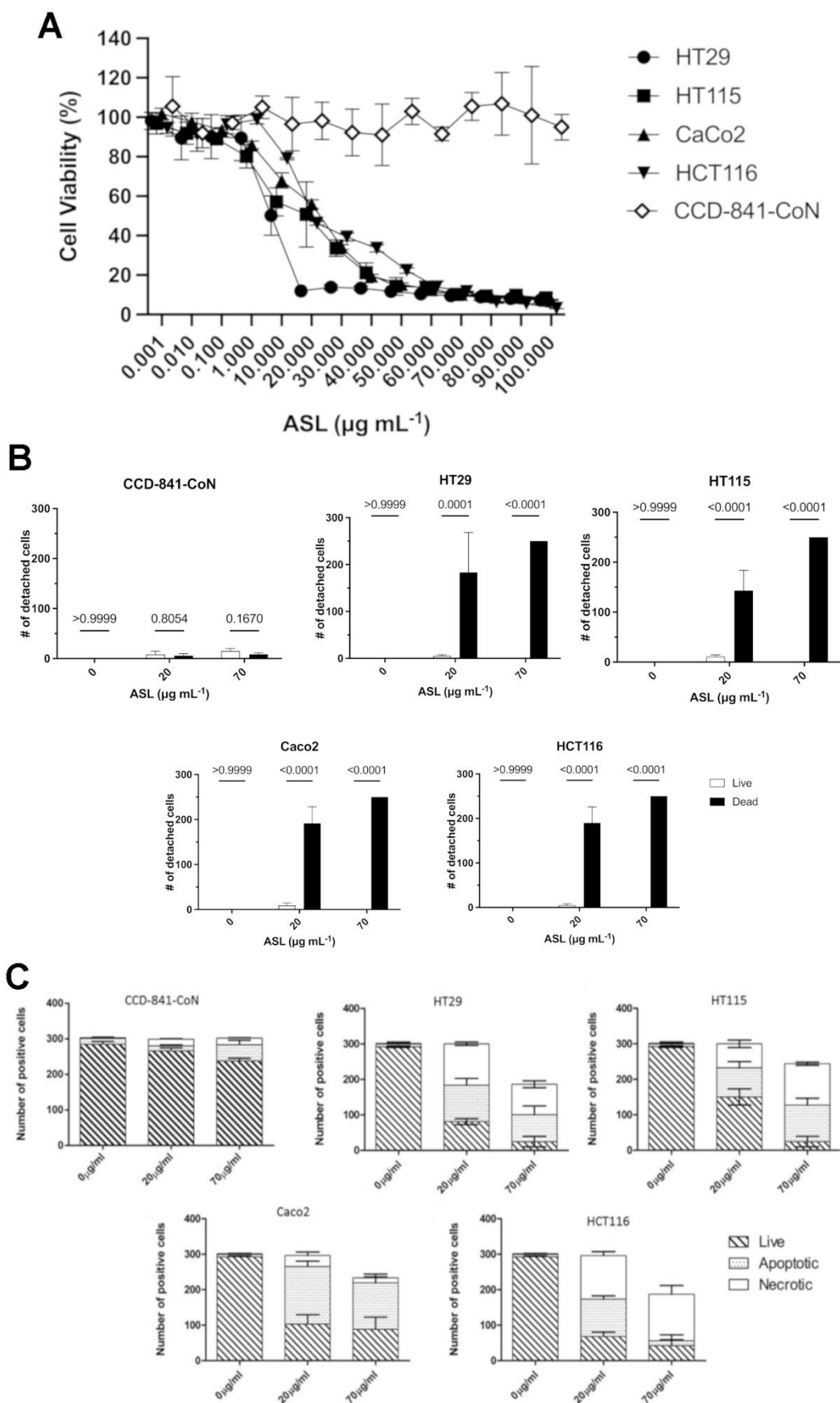
**Fig. 1.** Treatment with ASL results in a detrimental effect on colorectal cancer cell lines *in vitro*. Compared to normal colonic epithelial cells, the viability of four colorectal cancer cell lines was significantly reduced when treated with ASL (a). Treatment with ASL also resulted in increased cell detachment in colorectal cancer cell lines compared to normal colonic epithelial cells (b). Colorectal cancer cell lines treated with either 20 or 70  $\mu\text{g mL}^{-1}$  ASL showed indications of both apoptosis and necrosis driven cell death (c).

**Fig. 2.** Treatment with ASL significantly reduces cell migration in colorectal cancer cell lines. In a scratch assay ASL treated colorectal cancer cells showed significantly less migration 72 h post treatment than these treated with vehicle-only controls (a). Cell migration across a Boyden chamber in response to a stimulus was significantly reduced in colorectal cancer cells treated with ASL in comparison vehicle-only control treated cells (b). Two-way ANOVA (\*\*  $p < 0.01$  and \*\*\*  $p < 0.001$ ).

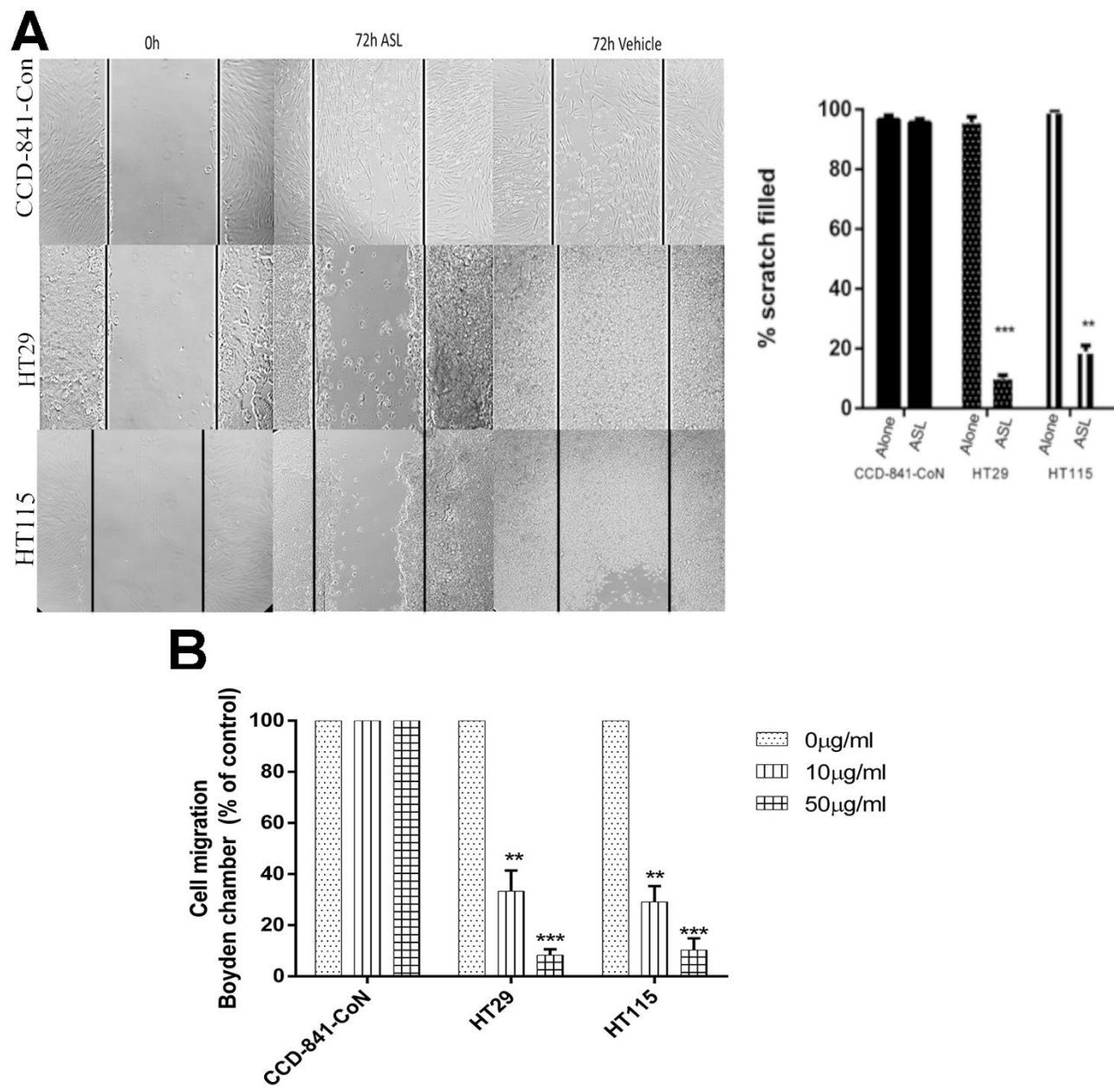
**Fig. 3.** An ASL supplemented diet reduced intestinal bleed in a mouse model of colorectal cancer. No morphological differences were observed in ileal sections of either wt or *Apc<sup>min/+</sup>* mice fed with either vehicle-only controls or ASL, however vehicle-only control fed *Apc<sup>min/+</sup>* mice showed evidence of intestinal bleeding from polyps (arrows), whereas ASL fed *Apc<sup>min/+</sup>* mice showed no evidence of intestinal bleeding (a). No significant difference in intestinal polyp number (b) or polyp sizes (c) was observed in *Apc<sup>min/+</sup>* mice fed on either

ASL or vehicle-only control. Following 35 days no significant difference was observed in haematocrit in  $Apc^{min+/-}$  mice fed with ASL or vehicle-only control (**d**) however, after 70 days a significant increase in haematocrit in  $Apc^{min+/-}$  mice fed with ASL was observed compared to those fed with vehicle-only control (**e**). One-way ANOVA,  $p$  values displayed on graphs.

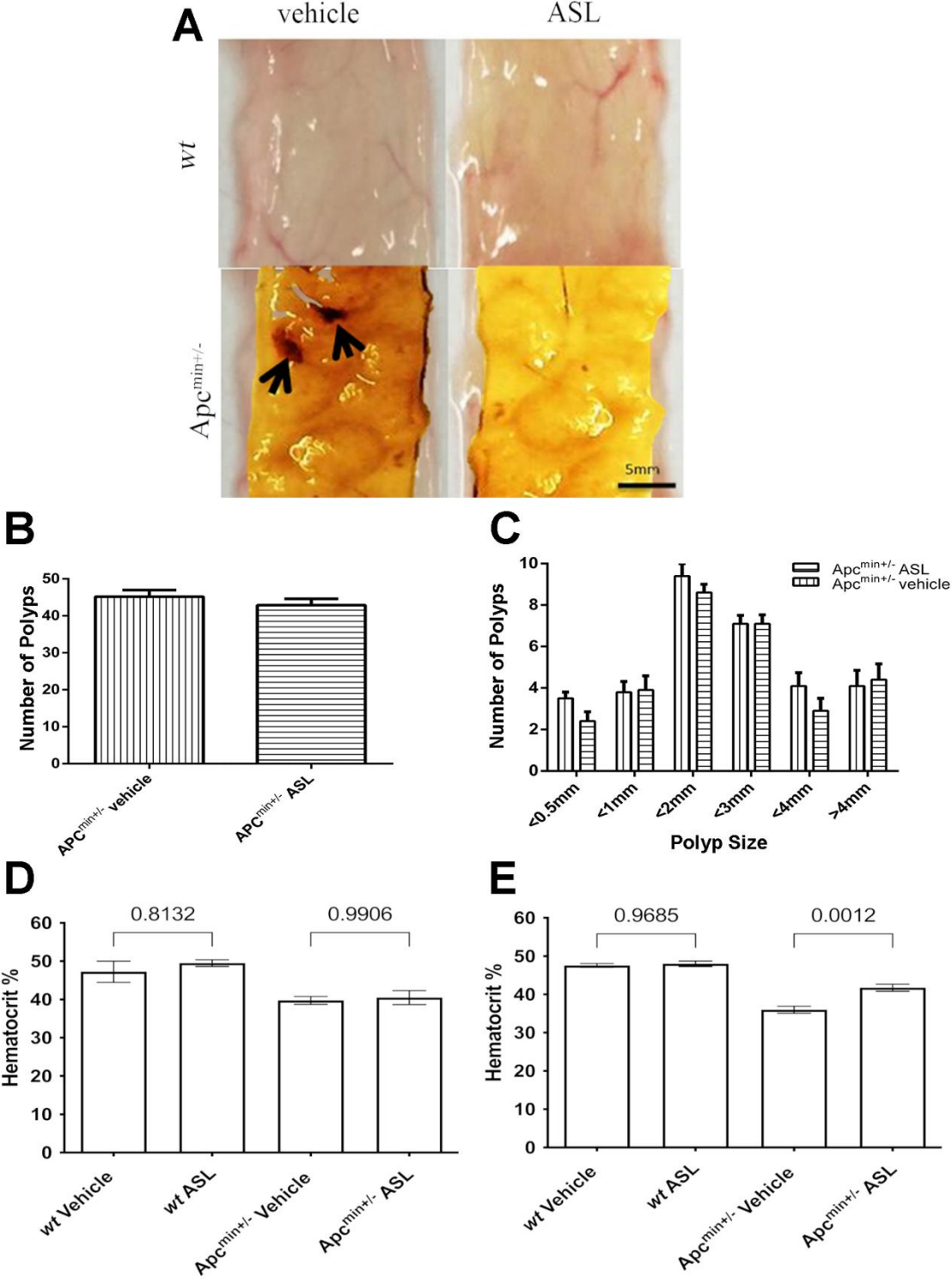
**Fig. 4.** Following 70 days, both splenic weight (**a**) and percentage of red pulp regions (**b**) in  $Apc^{min+/-}$  mice fed with ASL were significantly reduced in comparison to those fed with vehicle-only control ( $p < 0.001$  and  $p < 0.05$  respectively).  $Apc^{min+/-}$  mice fed with vehicle-only control had increased red pulp with a loss reticular structure compared to wild-type mice. Splenic morphology (particularly in white pulp) after feeding  $Apc^{min+/-}$  mice with ASL more closely resembled that seen in wild-type mice (**c**).



757 **Fig. 2.**



767 Fig. 3.



768

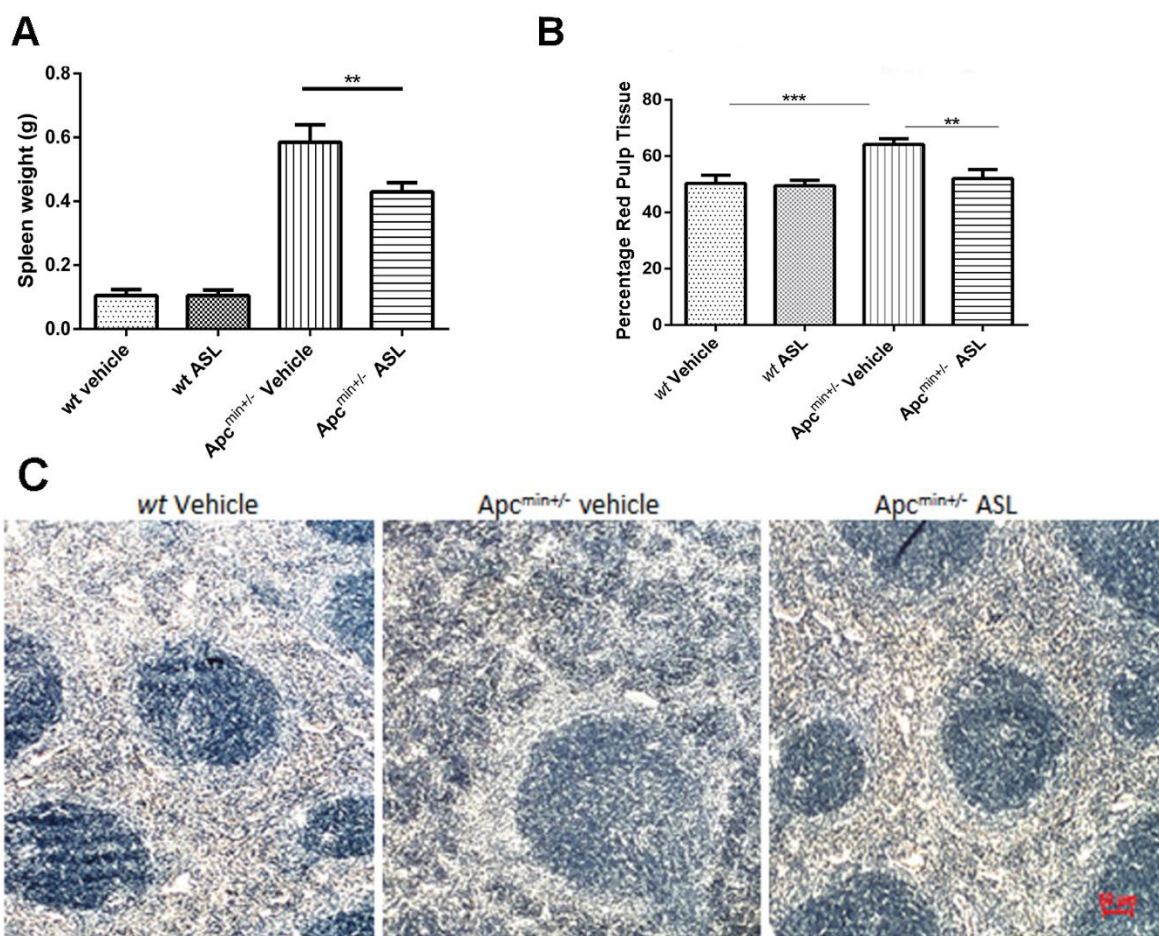
769

770

771

772

773 Fig. 4.



774



## Cell Death.tif

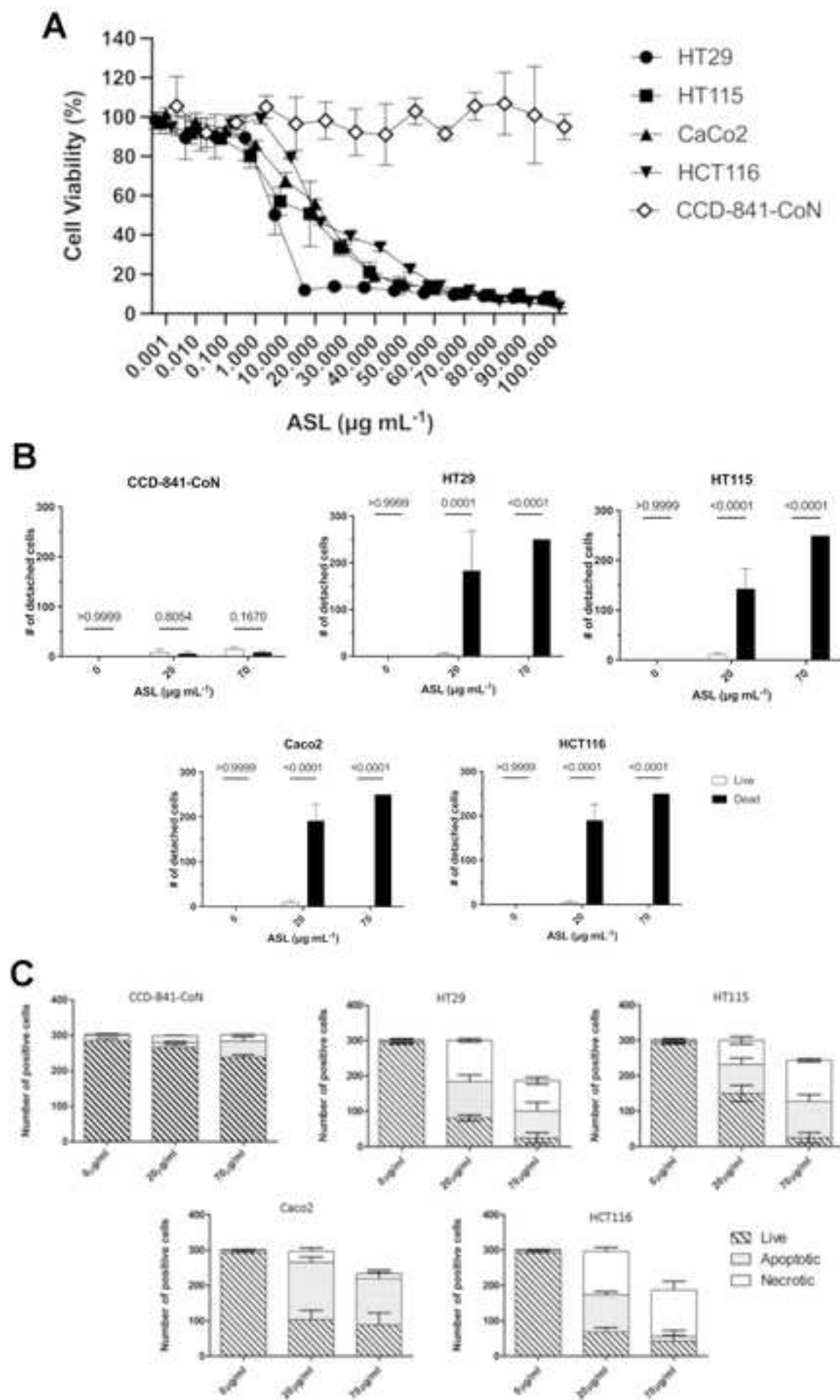




Fig2b\_BoydenChamber

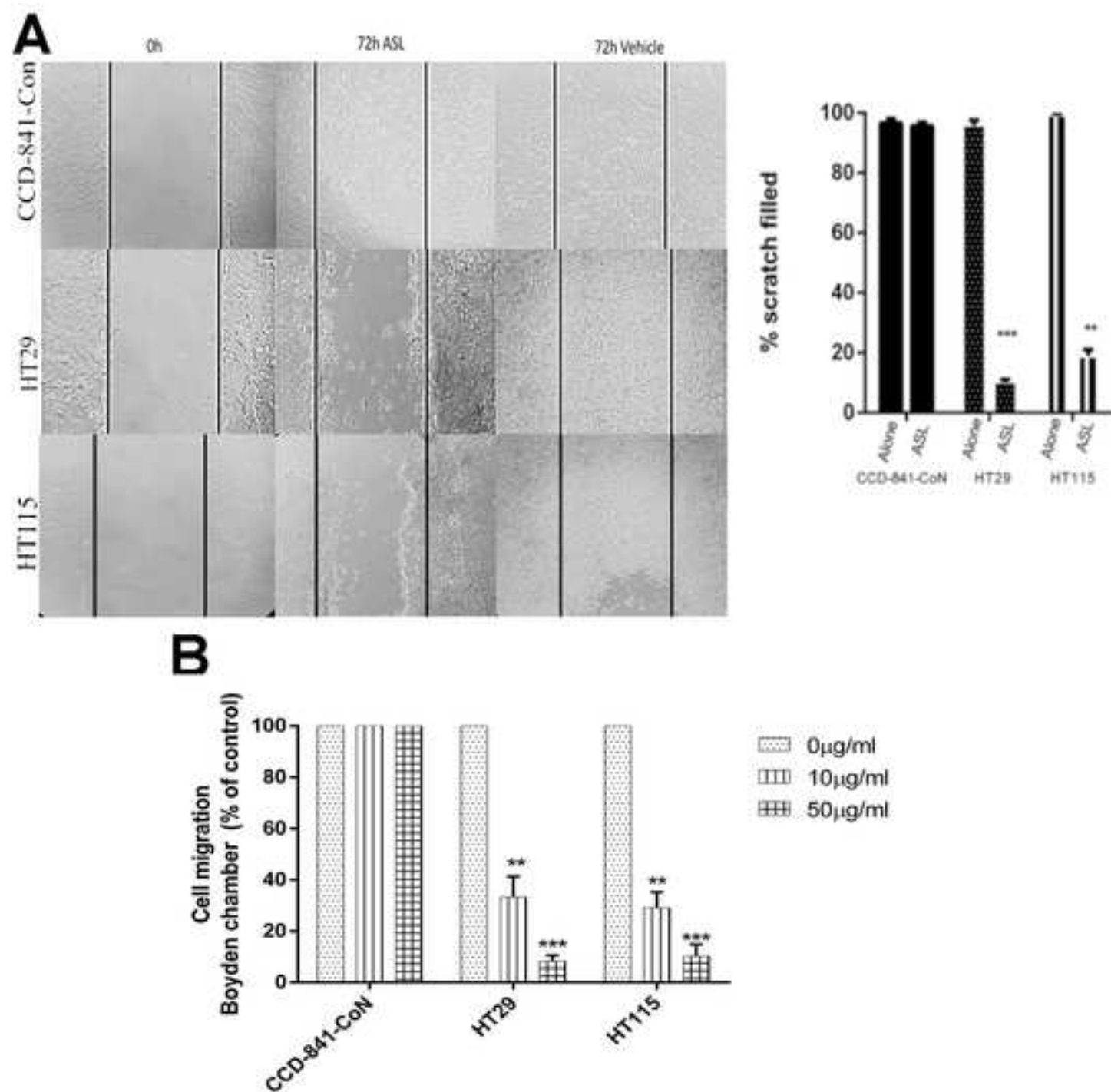
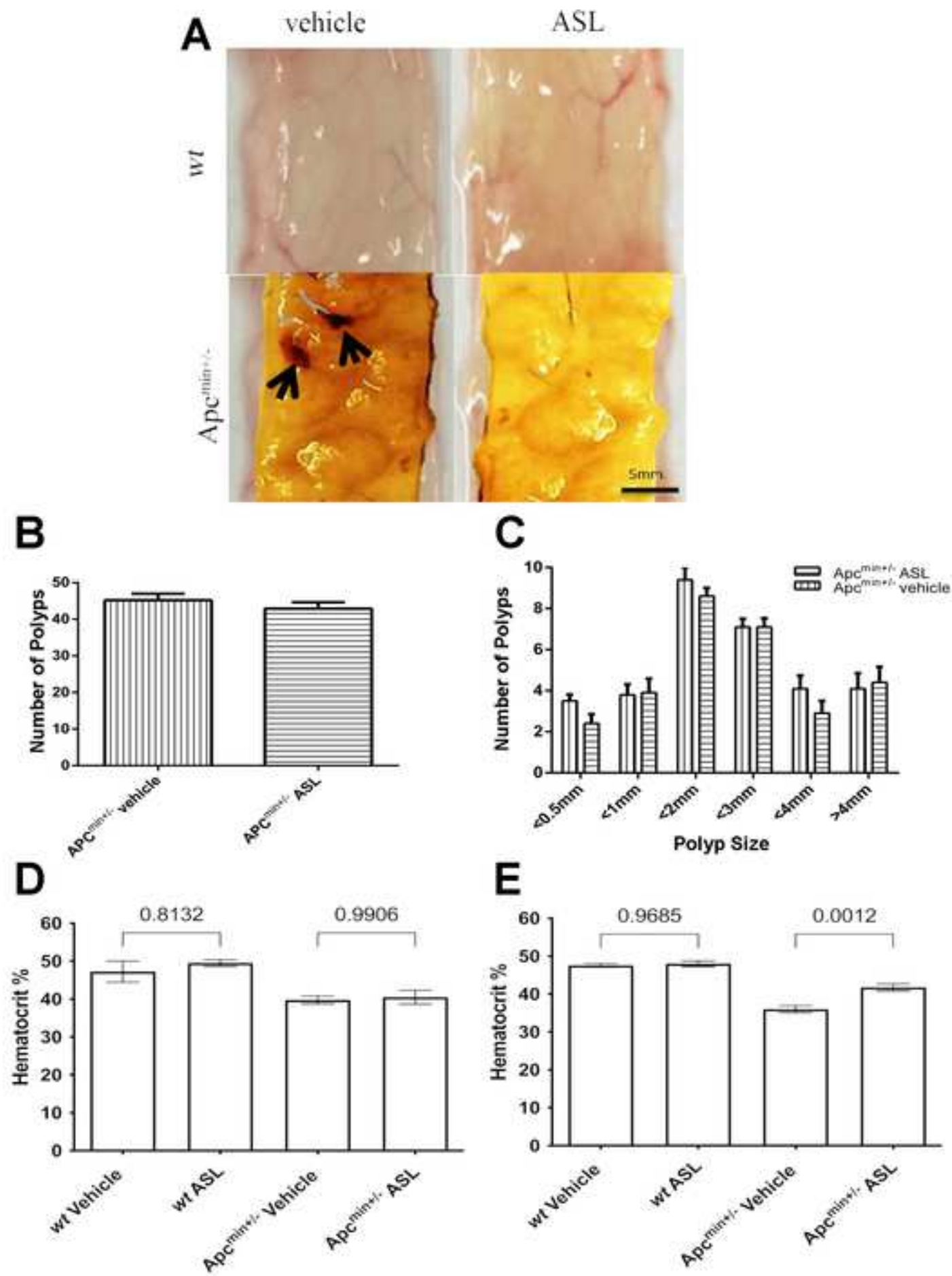
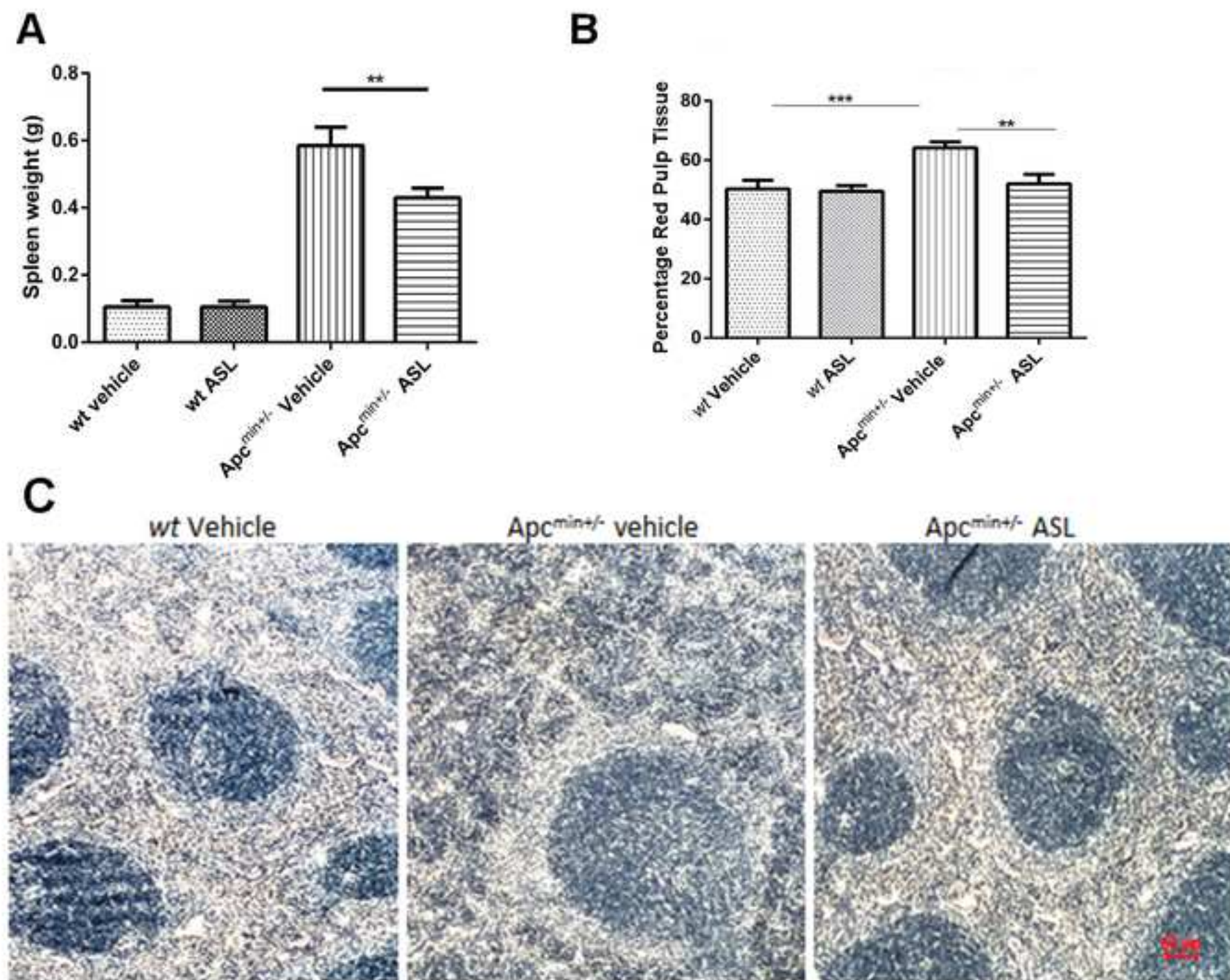


Figure 3

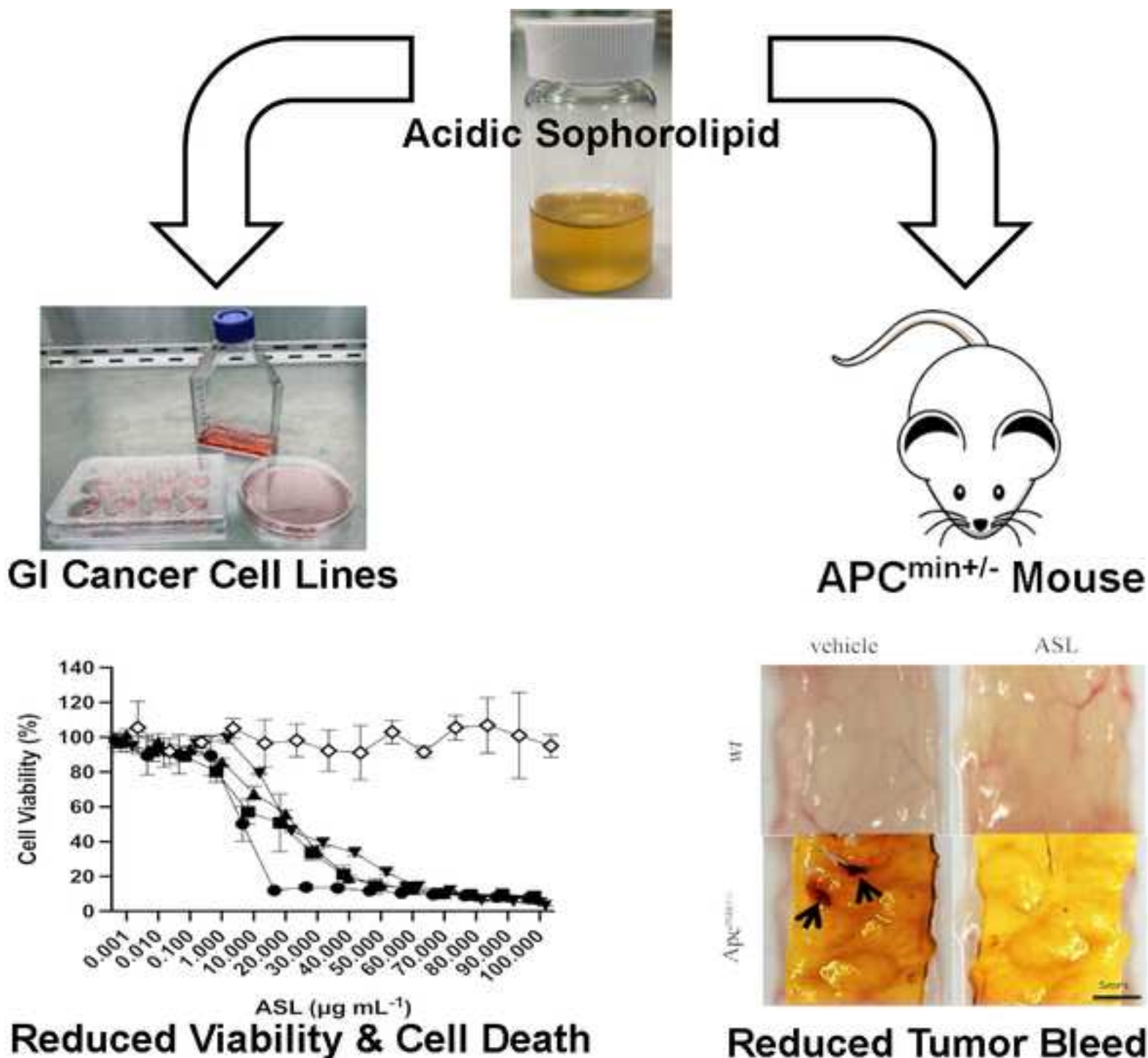
[Click here to access/download;Figure;Figure3\\_PolypData.tif](#)

Fig3c\_PolypSize











[Click here to access/download](#)

**Supplementary Material**

ASLpaper\_SupplementaryMaterial\_Clean.docx

