The hollow fibre model - facilitating anti-cancer pre-clinical pharmacodynamics and improving animal welfare

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Abstract. We describe a modified hollow fibre assay (HFA) for investigating the potential of novel molecules as pharmaceutical agents. In particular the assay provides drug/target interaction data that can facilitate the selection of lead compounds for further evaluation in more sophisticated solid tumour models, whilst successfully implementing the 3Rs - the 'replacement' 'refinement' and 'reduction' of animals. This more ethical and rapid approach to early drug development does not compromise on the validity, sensitivity, predictivity or efficacy of pre-clinical evaluation. We present novel data using the standard cross-linker mitomycin C (MMC) as a positive control, and two investigational DNA interactive molecules (C1311/ SJG-136). Tumour cells were seeded in fibres and implanted into mice. Following treatment with an intraperitoneal injection, fibres were excised and cells retrieved for pharmacodynamic analysis using the comet assay/fluorescence microscopy. Microscopy results revealed nuclear uptake and localisation within cytoplasmic organelles of HT29 colorectal adenocarcinoma cells following treatment with C1311 (150 mg/kg). Following treatment with SJG-136 (0.3 mg/kg) a 27.3% ($p<0.001$) DNA cross-linking (s.c.) effect was observed in the HL60 acute promyelocytic leukaemia cell line. DNA cross-linking effects of 55% (i.p) and 50% (s.c.) ($p<0.005$) were observed in the A549 lung carcinoma cell line following administration of MMC (6 mg/kg). These data are consistent with previous activity defined using solid tumour models, and support the use of the HFA for *in vivo* pharmacodynamic investigation whilst significantly reducing animal numbers and the influence of tumour growth on the welfare of mice.

Introduction

The pre-clinical testing of anti-cancer agents prior to clinical registration is steeped in a history of using considerable numbers of animals. The philosophy of the 'replacement, refinement and reduction' (3Rs) of animals in research first documented by Russell and Burch in 1959 (1) has influenced new legislation aimed at controlling the use of animals. The 3Rs have since been formally incorporated in the Animal (Scientific) Procedures Act, 1986 (UK) and the Animal Welfare Act, 1998 (USA). In September 2004 a national centre for the replacement, refinement and reduction of animals in research - 'NC3Rs' was established in the UK which aims to increase the development and implementation of the 3Rs in biomedical, biological and veterinary research in academia and industry.

Both the NC3Rs and the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) (USA) (2) aim to fulfil the need for considering the humane treatment of animals whilst maintaining scientific credibility. Despite the awareness of federal regulatory and research agencies [e.g. National Cancer Institute (NCI) and Food and Drug Administration] very little literature documenting precise methods in which to implement the 3Rs philosophy is available. We believe that adherence to the 3Rs philosophy should be a very important factor considered when planning animal experimentation, and we present the hollow fibre assay (HFA) as a means of implementing the 3Rs in early pre-clinical drug development.

The HFA was developed by the NCI as a low cost, high-throughput, preliminary *in vivo* screening assay for the evaluation of anti-cancer agents (3). It has since been adapted for the evaluation of antiviral agents (4), and there is considerable scope for use in other medical fields.

Since 1995 the NCI protocol has involved the short-term *in vitro* culture (24-48 h) of cells inside biocompatible hollow fibres, followed by implantation at both subcutaneous (s.c) and intraperitoneal (i.p) sites of the nude mouse (3 fibres/site). Mice are treated with test compound at two doses for up to 4 days, fibres excised and cells assessed for 'viability' using a modified MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) assay (3,5). Optimal or near-optimal treatment regimens are indicated for further testing using xenograft models (3).

Due to the practicality of being able to implant up to six different cell lines in each host (3/site) the NCI HFA requires only 24 mice for the testing of one agent against a panel of 12 cell lines. In contrast the NCI initially use three xenograft models to test one agent with each xenograft model requiring ~50 mice.

In line with the transition from the development of classical cytotoxic drugs to target-orientated drug discovery (6), more recently the HFA has been used to investigate the pharmacodynamics (drug-target interaction) of anti-cancer agents *in vivo*. Researchers are taking advantage of the practical feasibility

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of pure tumour cell retrieval from fibres and have used a variety of techniques to evaluate pharmacodynamic (PD) activity (7-11). In contrast, solid tumour models are not so amenable to pure cell retrieval due to the presence of host cells, and a laborious protocol is required before performing PD analysis. Recently the HFA has also been developed for studying agents that target the tumour neovasculature (12). The HFA was not developed to recapitulate the complexities of human cancer or to reproduce the complex interactions of the host cell/tumour micro-environment. It is proposed as an ideal *in vivo* method in which to demonstrate proof of principle activity or indeed to eliminate agents not shown to interact with their respective targets *in vivo* at an early pre-clinical stage. Agents identified as active in the HFA may progress and undergo more comprehensive pharmacokinetic/PD studies using xenograft models, but this initial evaluation using the HFA will still lead to considerable savings in animal numbers.

We describe novel pharmacodynamic end-points of the *in vivo* HFA, namely DNA cross-linking and drug uptake/cellular localisation evaluated using the COMET assay and fluorescence microscopy. Both standard and investigational agents, MMC, SJG-136, and C1311 will be used.

Mitomycin C is a DNA cross-linking agent that has been used in the clinic for over 20 years. It is used as a primary chemotherapy agent in the treatment of anal, lung and superficial bladder cancers and also as a secondary agent in breast, colon, gastric and pancreatic cancers.

SJG-136 is a recently synthesised pyrrolo[2,1-c][1,4]benzodiazepine (PBD) dimer currently undergoing phase I clinical trial in both the UK (CRUK) and USA (NCI). The pyrrolo[2,1-c][1,4]benzodiazepines are a family of tricyclic antitumour antibiotics which interact sequence selectively with purine-guanine-purine motifs in the minor groove of DNA, reacting covalently with the exocyclic C_2-NH_2 of the central guanine via their electrophilic imine moiety (13).

C1311 is a member of a group of imidazoacridinones that have been developed in an attempt to generate antineoplastic agents combining the common characteristics of acridines and anthracyclines essential for anti-cancer activity (e.g. planar structure and polycyclic nucleus) with high affinity to target DNA and resistance to production of free radicals by enzymatic reduction (14). Ultimately we aim to demonstrate the use of the HFA for PD investigation *in vivo* whilst successfully implementing the philosophy of the 3Rs.

Materials and methods

Cell culture. All cell culture procedures were carried out in sterile microflow class II biological safety cabinets (MDH). A549 lung carcinoma (15), HL60 acute promyelocytic leukaemia (16), and HT29 colorectal adenocarcinoma (17) cell lines previously confirmed as *mycoplasma*-free, were cultured using RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO) containing 10% fetal calf serum (FCS) (Sigma-Aldrich-Aldrich), 1% penicillin/streptomycin (50 μg/ml) (Sigma-Aldrich), 1% L-glutamine (2 mM) (Sigma-Aldrich), and 1% sodium pyruvate (1 mM) (Sigma-Aldrich). Cells were grown in 75 cm^3 flasks (Costar® High Wycombe, UK) in a humidified incubator at 37˚C with 5% CO_2 (Heraeus Instruments, South Plainfield, NY).

Hollow fibre preparation. All hollow fibre procedures were based on those of Hollingshead *et al* (3). Polyvinylidine fluoride (PVDF) hollow fibres (Type f) (Spectrum® Laboratories Inc., Houston, TX) with a 500 kDa mw cut-off, and 1 mm inner diameter were used. Tumour cells were cultured as described above, a subconfluent flask of cells attained and seeded at the desired density into hollow fibres. Fibres were placed in a 75 cm^3 (Costar) flask of RPMI-1640 medium (20% FCS) and incubated at 37˚C (5% CO_2) overnight.

In vivo implantation of hollow fibres. Pure strain female NMRI mice (B&K Universal Ltd., Hull, UK) or NCR/Nu (NCI, Frederick, USA) mice aged 6-8 weeks were used. All mice had access to food (CRM diet, SDS, Witham, Essex, UK) and water *ad libitum*. All animals were kept in cages in an air-conditioned room with alternating cycles of light and dark. All animal studies were carried out under a Home Office licence. UKCCCR guidelines for the welfare of animals in experimental neoplasia were adhered to throughout the study (18). Mice were anaesthetised by inhalation of isoflurane (Rhodia Organique Fine Ltd.). Three fibres were implanted at both subcutaneous (s.c.) and intraperitoneal (i.p.) sites. Three mice were used per treatment group. Incisions (i.p. and s.c.) were closed with histoacryl tissue glue (B/braun Surgical, Barcelona, Spain), with an additional skin staple.

Tumour cell retrieval from hollow fibres. Twenty-four hours following treatment mice were sacrificed by cervical dislocation, fibres excised, wiped to remove excess host tissue and transferred to pre-warmed RPMI (10% FCS) medium for 30 min. Fibres were transferred to 1 ml of pre-warmed accutase solution (Innovative Cell Technologies; in Dulbecco's phosphate buffered saline 0.5 mmol/l EDTA) in 6-well plates. Fibre ends were cut and discarded. Fibres were flushed through once with the 1 ml accutase solution. Plates were then incubated at 37˚C (5% CO_2) for 30-45 min on an orbital shaker at 100 g (Lab-Line Instruments, Melrose Park, IL) to detach cells from fibre walls and attain a single cell suspension ready for the comet assay or microscopy analysis.

Assessment of tumour cell growth within hollow fibres. Prior to performing pharmacodynamic investigations tumour cell growth within hollow fibres was characterised so that linear growth could be defined. On each day of growth assessment mice were sacrificed by cervical dislocation, fibres excised, and a modified version of the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (tetrazolium) dye conversion assay performed (3). Absorbance was measured at 540 nm using a spectrophotometer (Multiscan Plus, Labsystems, Life Sciences International Ltd., Basingstoke, UK., Genesis Labsystems software version 3.04). The percentage of net cell growth was calculated as follows:

$$\frac{\text{Mean absorbance on retrieval day - Mean absorbance on implantation day}}{\text{Mean absorbance on implantation day}} \times 100$$

Chemotherapy. Mitomycin C (M.W. 334.3) (Sigma-Aldrich), and C1311 (M.W 350) (Xanthus Life Sciences) were dissolved in physiological saline and administered at previously defined

maximum tolerated doses (MTDs)/therapeutic doses of 6 mg/kg (19) and 150 mg/kg (20,21) respectively.

SJG-136 (M.W 557.1), a gift from Professor D. Thurston (School of Pharmacy, University of London, UK) was dissolved in 5% dimethyl-acctamide/saline and administered at a known therapeutic dose of 0.3 mg/kg (22). All compounds were given 0.1 ml per 10 g bodyweight by a single intraperitoneal injection.

Alkaline single cell gel electrophoresis assay (Comet Assay). Comet assay procedures were based on those of ref. 23. At specific time-points following treatment with MMC/SJG-136 mice were sacrificed, fibres excised and tumour cells prepared as described above for analysis. Mice were sacrificed 24 h and 2 h following treatment with SJG-136 and MMC respectively as MMC-induced DNA cross-links are known to be repaired relatively rapidly compared to SJG-136-induced DNA cross-links which are thought to be irreversible. Each cell suspension was pelleted and incubated with H_2O_2 (100 μM) (4˚C), (20 min) (24) to introduce single strand breaks. Cell suspensions were pelleted and resuspended in 150 μl PBS. Many electrophoretic methods for the separation of DNA containing an interstrand crosslink are based on the principle that such a lesion can prevent the complete denaturation of the two DNA strands. Therefore as the number of crosslinks increases the amount of DNA able to migrate is reduced. Thus crosslinks are quantified as the decrease in comet tail moment compared with untreated controls.

Frosted edge slides were placed into normal melting point (NMP) agarose (1%) (45˚C), removed, the underside wiped and allowed to dry overnight. All procedures from here were carried out in a dark room under red light. Low melting point agarose (150 μl) (LMA) (1%) (45˚C) was added to each cell suspension. Each cell suspension (150 μl) was added to two agarose coated slides, a coverslip placed on each, and slides placed on ice for ~5 min. The coverslip was removed and 150 μl of LMA (0.5%) was added as a third layer for protection of cells. The coverslip was replaced and the slide placed again on ice for 5 min.

Coverslips were removed and all slides gently placed in a slide rack in cold-lysis solution (2.5M NaCl, 100 mM ethylene-diaminetetraacetic acid (EDTA), and 10 mM Trizma®Base pH 10.0) for a minimum of 1 h (4˚C). Dimethyl sulfoxide (10%) (DMSO) and 2 ml of Triton X-100 was added to lysing solution. Slides were placed in a horizontal electrophoresis gel tank and covered with electrophoresis buffer (1 mM EDTA and 300 mM NaOH, pH >13.0), and incubated for 30 min to unwind DNA. DNA was then electrophoresed at 25 V for 30 min (CONSORT 600 V-1000 mA E861).

Slides were removed and washed with neutralizing buffer (TrizmaBase pH 7.5) for 5 min. Slides were covered in PBS for 5 min. Fresh PBS was added for an additional 5 min. Slides were air-dried overnight, and placed in distilled water for 30 min.

Slides were stained with SYBR®gold (x10,000 in distilled water) (Molecular Probes, Carlsbad, CA) (500 μl/slide) and incubated for 30 min. Slides were placed under a fluorescence microscope (LEICA DMLB). Using Comet III - Perceptive Instruments (Suffolk, UK) software, connected to the fluorescence microscope and camera, cells were counted and analysed. Cells (25) were counted randomly from one slide and another 25 from the replicate slide.

Tail moment (measurement of tail length x measurement of DNA in the tail) was used as a primary measure of DNA damage. Percentage DNA cross-linking was determined using the following calculation:

100 - (mean treated comet value/mean control value x 100)

Fluorescence microscopy. At 4 h, mice were sacrificed by cervical dislocation, fibres excised and prepared as described above. Single cell suspension was pelleted, resuspended in RPMI medium, and pipetted onto to glass slides and a coverslip placed on top. Images of C1311 cellular uptake and localisation were captured using a confocal microscope (Biorad Microradiance Confocal Imaging System) attached to a Nikon CM-800 microscope using Laser Sharp 2000 software (excitation/emission maxima 340/520 nm).

Tissue processing and staining of fibre sections. Fibres were retrieved from mice and placed in 10% buffered-formalin for ~4 h, and transferred to 70% ethanol until required for processing. Fibres were embedded in paraffin in a Leica TP 1020 automatic tissue processor (Leica, Milton Keynes, UK). Fibres were removed from the tissue processor, aligned in small plastic cubes and filled with hot paraffin wax on an embedding centre (Raymond Lamb, East Sussex, UK). Cubes were placed on a cold plate and left over night to solidify before being sectioned. Sections (10 μm) were cut using a rotary microtome, mounted on glycerine albumen (Merck) coated slides, and allowed to dry overnight. Slides were submerged sequentially in haematoxylin (10 min), (washed, tap water), acid alcohol (0.5% hydrochloric acid in 70% ethanol) (5 sec) (washed, tap water), Scott's tap water (2 min), 1% aqueous eosin (1 min) (wash), 100% ethanol (1 min), ethanol (3 min), 50% xylene/ethanol (3 min), 100% xylene (3 min) and 100% xylene (5 min). Several drops of DPX were added to a coverslip, placed on each slide, and allowed to air dry. Slides were then ready to be observed under the light microscope.

Results

Characterisation of tumour cell growth within hollow fibres in immuno-competent and immuno-deficient hosts. We present data demonstrating that human tumour cells grow equivalently in both immuno-competent NMRI and immuno-deficient athymic nude hosts (Fig. 1). HT29 colorectal adeno-carcinoma cells were seeded in hollow fibres and implanted in both hosts over seven days. At each time-point mice were sacrificed, fibres excised and the MTT assay performed to assess cell viability. Raw absorbance values derived from fibres (3/site) retrieved from both hosts (s.c/i.p) were compared at each time-point. No significant difference ($p>0.05$) in raw absorbance values was observed between NMRI immuno-competent and nude immuno-deficient mice on days 3, 5 and 7 (i.p/s.c). Fig. 1 presents mean percentage net cell growth data derived from raw absorbance values. Fig. 1 insert shows the morphology of HT29 cells growing along the internal surface of the fibre *in vivo*.

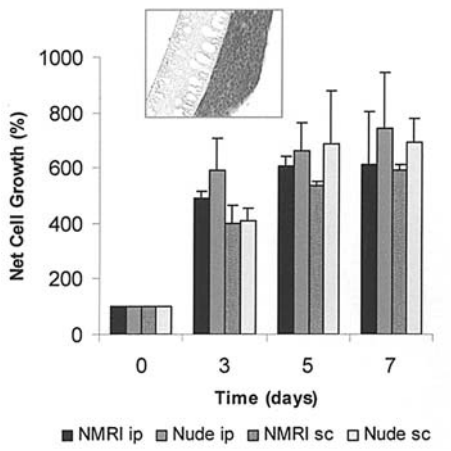

Figure 1. Net cell growth (%) of HT29 colon carcinoma cells grown in hollow fibers implanted in nude and NMRI mouse strains. Tumor cells were seeded at 1xl0^6 cells/ml in hollow fibers and incubated overnight (37°C) (5%, CO_2) in RPMI media (20% FCS). Fibers were implanted into mice at s.c. and i.p. sites. At each time-point mice were sacrificed, fibers excised and the MTT assay performed to determine cell viability. Results are expressed as mean % net cell growth. Insert, HT29 colon carcinoma cells growing along the internal surface of the fibre at the s.c. site in NMRI mouse.

DNA cross-linking assay. The Comet assay was used to assess the pharmacodynamic activity of the standard cross-linking agent MMC following treatment of NMRI fibre-bearing mice at the MTD. Fig. 2 presents frequency histograms of DNA comet tail moments and comet images following analysis of A549 non-small cell lung carcinoma cells retrieved from fibres 2 h post treatment. Mean tail moments calculated from MMC-treated mice were 31.96±25.56 and 31.5±23.12 at i.p. and s.c sites respectively. Mean values of cells retrieved for vehicle-treated mice were 71.49±32.1 and 62.3±30.28 at i.p. and s.c. sites respectively. Differences in tail moment distribution between control and treatment groups at both i.p and s.c sites are highly significant ($p<0.005$, Mann Whitney test). Percentage MMC-DNA cross-linking values were calculated at 55.29% and 49.44% for i.p. and s.c. sites respectively.

The pharmacodynamic activity of the investigational agent SJG-136 was also evaluated using the comet assay. Fig. 3 presents DNA frequency histograms of comet tail moments and comet images of HL60 leukemia cells retrieved from hollow fibres (s.c) 24 h following treatment with the MTD. HL60 cells retrieved from SJG-136- and vehicle-treated mice were shown to have mean tail moment values of 16±9 and 22±12 respectively ($p<0.001$ Mann Whitney test). A 27.3% DNA cross-linking effect of SJG-136 was calculated.

Fluoresence microscopy. NMRI fibre-bearing mice were treated with the imidazoacridinone C1311 (150 mg/kg). At 4 h after treatment, fibres were excised and HT29 cells retrieved for fluorescence microscopy analysis. Nuclear localisation was observed along with punctate concentrations evident in cytoplasmic organelles (Fig. 4).

Discussion

This study has successfully characterised the HFA for investigating novel pharmacodynamic end-points *in vivo*, namely DNA cross-linking and drug uptake/cellular localisation, whilst implementing the philosophy of the 3Rs.

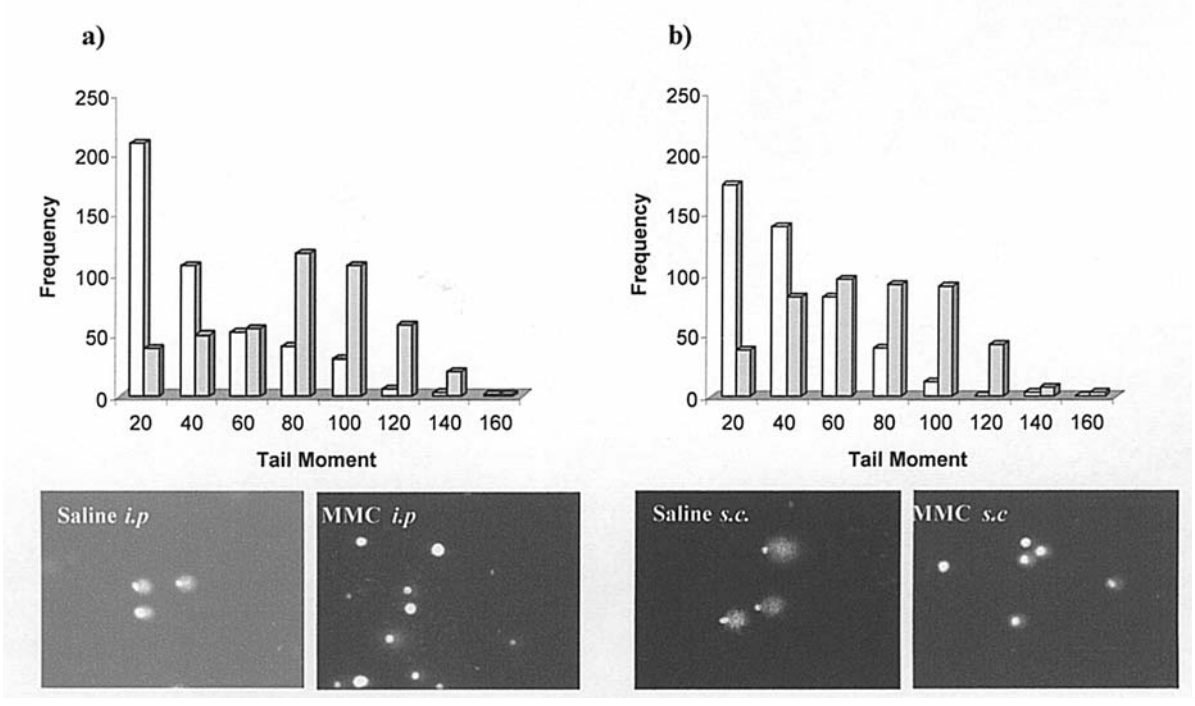

Figure 2. Frequency histograms of DNA comet tail moments. A549 non-small cell lung carcinoma cells were seeded in hollow fibers (1xl0^6 cells/ml), cultured *in vitro* overnight, and implanted into NMRI mice at both a, i.p; and b, s.c. sites. On day 3 mice were treated with either MMC (white bars) or physiological saline (grey bars) at 6 mg/kg by a single i.p. injection. At 2 h mice were sacrificed, cells retrieved from fibers and the comet assay performed. Each set of bars represents the analysis of 450 cells (3 mice x 3 fibers/site x 50 comets).

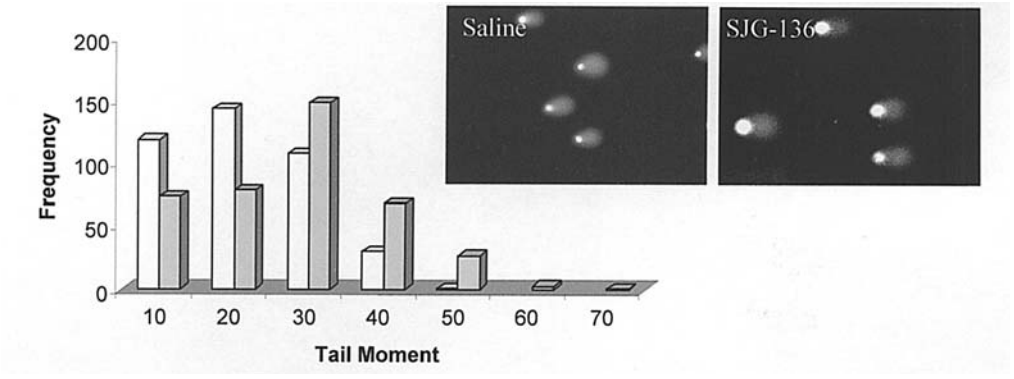

Figure 3. DNA frequency histogram of comet tail moments. HL60 leukemia cells were seeded into hollow fibers (1xl0^6 cells/ml) and implanted into NMRI mice at the s.c. site. On day 4 mice were treated with either SJG-136 (white bars) or 5% dimethyl-acetamide/saline vehicle (grey bars) at an MTD of 0.3 mg/kg by a single i.p. injection. At 24 h mice were sacrificed, cells retrieved and the comet assay performed. Each set of bars represents the analysis of 450 cells (3 mice x 3 fibers site x 50 comets).

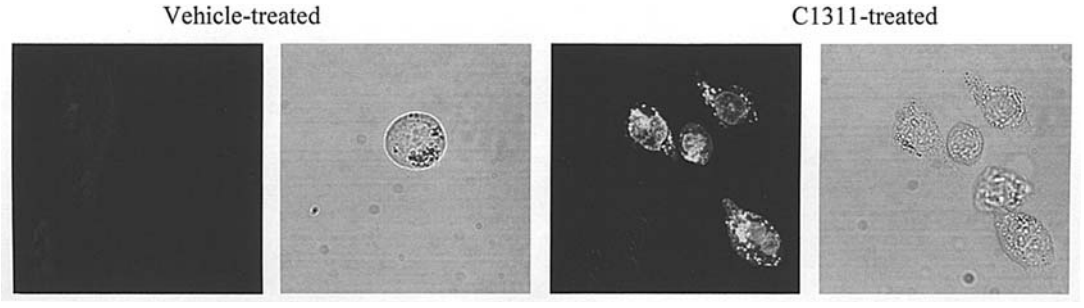

Figure 4. Fluorescence microscopy images: HT29 cells were seeded in hollow fibers (2xl0^6 cells/well), incubated overnight and implanted into the i.p. site of NMRI mice. On day 3 mice were treated with C1311 or physiological saline (150 mg/kg) (i.p.). Mice were sacrifled at 4 h, fibers excised and cells observed using fluorescence microscopy. Fluorescence images of HT-29 cells retrieved from vehicle-treated mice are also presented so that the effects of auto-fluorescence can be eliminated. Both fluorescence and transmission images are presented. Images are representative of whole tumor cell populations.

MMC has previously been found to be active using the HFA as defined by the routine MTT assay (9). Here the HFA has been used to confirm, for the first time the proposed PD activity of the standard agent MMC *in vivo*. This *in vivo* HFA activity supports previous activity defined using a breast xenograft model (25).

The PBD dimer SJG-136 has recently generated a score of 54/96 using the NCI routine HFA (22) and has demonstrated significant anti-tumour activity against several human tumour xenograft models including a HL-60 tumour model (26). Here the HFA has confirmed the proposed cross-linking activity of SJG-136 *in vivo*.

The cellular uptake and localisation *in vitro* (20,21,27) and activity in both murine and HT29 human tumour xenograft models (20) of the imidazoacridinone C1311 has previously been demonstrated. Rapid nuclear and lysosomal uptake was observed (27). Lysosomal swelling and breakdown have been shown to precede apoptosis, and it is thought that these lyso-osmotropic effects of C1311 may be a novel feature of this anti-cancer agent (27). C1311 has also previously been evaluated using the routine *in vivo* HFA, revealing a 68 and 57% growth inhibition in HT-29 cells respectively at the i.p. and s.c. site respectively (Personal communication: Jesse Paterson, Xanthus Life Sciences). Here we have used the HFA to demonstrate cellular localisation of C1311 for the first time *in vivo*, supporting previous *in vitro* and *in vivo* anti-tumour xenograft studies.

The results of our DNA crosslinking and drug uptake/cellular localisation pharmacodynamic studies collectively demonstrate the comparable activity generated in both HF (using 3 mice/group) and previous xenograft models.

Our laboratory has also demonstrated the selective PD activity of 'Phortress', a DNA reactive 2-(4-amino-3-methyl-phenyl)-5-fluorobenzothiazole prodrug currently in phase I trials, using both HFA and xenograft models (8). PD activity has also been demonstrated using Combretastatin A-1 phosphate (CA1P) in the HFA *in vivo* (7), supporting previous anti-tumour activity demonstrated using xenograft models (28). Known clinically active antiviral agents have been shown to suppress infection in both HFA and xenograft models (4), again demonstrating the comparable data defined by both xenograft and the HF models.

With respect to the development of cytotoxic agents, the traditional s.c. xenograft model appears to have value as a predictive *in vivo* preclinical model (29), yet it remains to be seen if the xenograft will retain its predictive capacity in this modern era of rationally designed small molecules. Whether alternative models (e.g. genetically engineered/transgenic/knockout) may be more predictive of clinical response or provide a more clinically relevant model is yet to be elucidated (30).

The HFA has consistently been shown to have good predictivity of xenograft activity (6,31,32). All agents that have progressed to the clinic for routine use that have been

tested in both xenograft and HF models have been found to be active in both models. Additionally, agents under clinical investigation identified as active in the NCI routine HFA, have also been tested extensively in xenograft models (e.g. Phortress, C1311, SJG-136, and the proteosome inhibitor PS341).

In recognition of the heterogeneity of clinical disease, it is believed that for a pre-clinical model to predict clinical activity it must possess the relevant molecular drug-target. Agents identified as active in molecularly characterised pre-clinical models must be rationally administered to patient populations possessing the relevant biomarker. Considering that many non-toxic therapies may be cytostatic it has been suggested that early phase clinical trials may need to incorporate measures of anti-tumour efficacy other than tumour size, for instance measurement of target inhibition (33). Parulekar and Eisenhauer's review of 60 recent phase I trials of non-cytotoxic agents clearly indicated that these data were lacking.

If drug-target interaction is to be considered as a biomarker in the clinic then it seems plausible to use a practically amenable *in vivo* model, namely the HFA (appropriately characterised for the relevant PD end-point) as a predictor of clinical activity. Such application may even speed up the selection of lead compounds for clinical trial.

Such pharmacodynamic application of the HFA permits the follow-up of a compound's mechanisms *in vivo* following its indication *in vitro* by molecularly characterised *in vitro* screens.

With respect to the 3Rs, implementing the principle of 'reduction' has caused concern with respect to using too few animals that could lead to inconclusive results (34,35). It is emphasised that each *in vivo* hollow fibre study uses only a maximum of 6 mice (3/group) permitting the retrieval of 9 individual tumour cell populations (3 i.p./s.c. x 3 mice) per group. In comparison only 1-2 individual tumour populations are derived from one xenograft mouse model. In the case of our comet assay analysis, a treatment group of 3 mice generates 450 comet tails moments (9/site x 50 comets) used for determination of mean tail moment. These numbers are sufficient to measure statistical significance.

If we take into consideration that one xenograft model would generally use 50 mice and that the HFA has determined activity comparable with that determined in xenograft models then it would seem plausible to rely on the HFA for such pharmacodynamic investigation. Simultaneously animal numbers are reduced significantly without compromising on the scientific credibility of the assay. As well as the considerable reduction in the numbers of mice required, other 3R benefits of the HFA include a shorter assay time of less than one week. In contrast routine xenograft models may take between two weeks and four months to complete depending on the end-points selected for study. Such a 'refinement' reduces the time animals are exposed to experimental procedures, and decreases the tumour burden thus reducing the release of cachectic and other deleterious material from tumour cells into the host.

These novel pharmacodynamic investigations that have successfully provided 'proof of principle' of a compounds mechanisms of action *in vivo* have been facilitated by the practical amenability of cell retrieval from fibres.

The debatable value of other *in vivo* models in predicting clinical response, evidence of good pre-clinical predictivity of the HFA, the comparable applicability of the HFA with the xenograft model, all lend support for the more extensive use of the HFA.

In this era of rational drug design the hollow fibre model if designed appropriately offers an amenable, rapid, cost-effective and ethically acceptable model in which to investigate unlimited drug-target interaction/target inhibition pre-clinically.

The implantation of hollow fibres has been demonstrated in both immunocompetent (36-38) and immunodeficient mouse strains (9-11,39,40) without causing immune destruction by the host or impeding the growth of tumour cells. Here we have demonstrated the comparable growth of tumour cells in hollow fibres *in vivo* in both immuno-competent and immuno-compromised hosts. These data confirm that various mouse strains may be used for such pharmacodynamic investigations which may also provide an economic advantage.

Although we do not believe that the HFA will replace more sophisticated animal models (e.g. transgenic/orthotopic/autochthonous/xenograft) we conclude that the hollow fibre model provides a valid method for investigating PD activity *in vivo* whilst successfully implementing the principles of 'reduction', 'refinement' and 'replacement', and will aid in the selection of lead molecules for further pre-clinical testing. We anticipate that the HFA will be used more readily in future years not only for reasons of amenability for PD investigation but for ethical reasons. Specifically the dramatic reduction in mouse numbers required, at a time when scientific researchers and the general public are becoming increasingly conscious of animal welfare.

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