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SHORT TALKS

New scientists

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Disintegration of oral controlled release formulations in the canine GI tract evaluated using gamma scintigraphy

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In-vivo evaluation of an oral modified release formulation is an important step to confirm performance as predicted by in-vitro tests. Previous studies have demonstrated the application of gamma scintigraphy to the beagle dog model (McInnes et al 2005), to allow visualisation of location and behaviour of simple oral capsule and liquid doses. The objective of the current work was to evaluate the transit and disintegration of two oral controlled release dosage forms using gamma scintigraphy. Two controlled release tablet formulations were prepared of the same drug (X) at different dosage strengths, using either powder blend (Formulation A = 20mg) or wet granulation (Formulation B = 100mg) processes. Formulation A was designed to release drug over 6 hours and consisted of HPMC (Methocel), lactose, Aerosil 200 and magnesium stearate. Formulation B was designed to release drug over 18 hours, and contained HPMC (Methocel), lactose, povidone (Kollidon) and magnesium stearate. 6 mg of enriched samarium oxide (97% ^{152}Sm) was added immediately before direct compression into circular convex tablets. When required for study the tablets were irradiated in a neutron flux for 2.5 min, converting the inactive ^{152}samarium to the gamma emitting radioisotope ^{153}samarium, providing activity of approximately 1MBq at time of dosing. Dissolution testing in pH 7.5 buffer was carried out before and after irradiation. For in-vivo studies, a gamma camera equipped with a low energy collimator was used to obtain posterior images at 2-min intervals until gastric emptying, every 15 min until colon entry, then every 30 min until the end of the study. External markers containing 0.1 MBq ^{99m}technetium were used for positioning. The scintigraphic images were used to assess time and location of initial and complete tablet disintegration. All procedures were performed under a UK Home Office Animals (Scientific Procedures) Project Licence, with free access to water allowed during the study, and food allowed when the radioactivity had entered the colon. Significantly different in-vitro release profiles were obtained for the two formulations ($F_2 = 33$), with formulation A releasing drug more rapidly than formulation B ($T_{80\%}$ release of 406 and 987 minutes respectively). Neutron activation did not have a significant effect on the profiles ($F_2 > 50$). Table 1 displays the mean in-vivo tablet disintegration times and location determined using gamma scintigraphy. The time to onset of disintegration was similar for both formulations, however the time to complete disintegration of formulation B was much shorter than for formulation A, a reversal of the of the in-vitro dissolution behaviour (differences not significant due to high inter-animal variability). The general trend was that disintegration of formulation A tended to occur throughout the GI tract, whereas it was towards the upper GI tract for formulation B. Using gamma scintigraphy it was determined that the in-vivo performance of the two formulations did not correspond with the in-vitro behaviour. The study demonstrated that in-vivo disintegration and location could be assessed in the beagle dog, and that the technique may be useful in the pre-clinical evaluation of modified release formulations.

Table 1 Number of tablets (n) against location/time of initial and complete disintegration

Formulation	Time ± s.d. (min)	Stomach (n)	Small intestine (n)	Caecum/Colon (n)
A				
Initial	45.4 ± 14	2	1	1
Complete	339 ± 181	1	1	2
B				
Initial	47.4 ± 40	3	1	
Complete	229 ± 171	2	1	1

McInnes, F. et al (2005) *J. Pharm. Pharmacol*. **57**: s225

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The mode of action of yeast mediated drug delivery

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The oral route of drug delivery is acceptable and convenient. However, problems of some drugs delivered via this route include poor adsorption in the gastrointestinal tract and degradation by gastric acidity. Therefore, encapsulation of drugs within a protective structure, a microcapsule, can enhance bioavailability and protect the drug from the harsh acidity of the stomach, releasing the drug further down the digestive tract where pH is neutral. Penetration of hydrophilic drugs across intestinal cells is controlled by tight junctions, which exist between one cell and its neighbour in an epithelium, forming an effective impenetrable barrier. Some microcapsules are designed to act by reversibly opening epithelial tight junctions, allowing increased paracellular drug transport. Previous work has shown that chitosan microcapsules, based on a deacetylated form of chitin, present both in crustacean shells and fungal cell walls, can increase the permeation of drugs across *in vitro* cell monolayer models by opening tight junctions (Smith et al 2004). Yeast cells (*Saccharomyces cerevisae*) can absorb and retain molecules (Bishop et al 1998) and previous work has demonstrated the reversible opening of epithelial tight junctions stimulated by yeast cells (Fuller et al 2005). It was therefore the aim of the present work to investigate the ability of heat-killed yeast cells to encapsulate model drugs (ibuprofen and propranolol hydrochloride) to allow improved drug delivery across a cell membrane and to elucidate the mode of yeast action at a cellular level. Model drugs were encapsulated within yeast cells using standard mixing and spray drying techniques. Drug loading levels were determined through extraction of drug and subsequent HPLC analysis. An increase in permeation of both drugs was observed across a model intestinal cell culture system (Caco-2 cells) using trans-epithelial electrical resistance (TEER) measurements of tight junction opening and HPLC analysis with subsequent P_{app} (apparent permeability) assessment of drug transport. Visualisation of drug transport across Caco-2 cells was achieved using fluorescent molecules and confocal microscopy to investigate the transport pathways involved. Yeast enhancement of epithelial permeability was elucidated using immunofluorescence microscopy of the tight junction associated proteins, ZO-1 and occludin, as well as employing inhibitors of cell signalling secondary messengers. Yeast cells were successfully used to encapsulate both drugs with good loading yields. The yeast microcapsules were shown to open tight junctions in a time- and dose-dependent manner, which was fully reversible and was non-toxic. There was an increase in Caco-2 cell permeability following application of yeast for 24 h, in terms of a decrease in TEER (Table 1) and at least a 10.8-fold increase in apical to basolateral transport of drugs, compared to drug transport in the absence of yeast (Table 1). Yeast-mediated cell permeability involved translocation of ZO-1 and occludin from the membrane to the cytoplasmic area of the cell, through PKC and protein tyrosine phosphatase cell signalling second messengers. In this study we demonstrated that yeast cells can be used as effective microcapsules for the delivery of drugs in a model system. Indeed, the unique properties of the yeast cell surface act to enhance drug absorption by modulating epithelial tight junctions.

Table 1 Yeast mediated decrease in TEER and increase in Papp of two drugs compared to control over 24 h (0% yeast)

Test suspension	TEER (% of baseline)	P_{app} ($\times 10^{-6}$ m/s)	
		Ibuprofen	Propranolol
2% yeast	53.5% ± 4.21	3.35 ± 6.84	3.82 ± 5.30
0% yeast (control)	101.2% ± 2.24	0.31 ± 6.09	0.34 ± 4.89

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Pgp-dependant "vacuum cleaner" functionality is potentially ruled by endocytosis

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Multi drug resistance (MDR) is a phenomenon whereby living organisms develop broad resistance to a wide variety of therapeutic drugs not structurally related. MDR is associated with over-expression of transmembrane transporters acting like "inverted flippase" by extruding drugs from the plasma membrane inner leaflet of living organisms, before they diffuse within. Strikingly, MDR is efficiently generated with a small amount of these transporters (Mao & Scarborough 1997). As therapeutic drugs usually partition efficiently into membranes, their insertion is likely to be widespread and there are no reasons why a drug should insert into membranes in the vicinity of a transporter. As a result, it is suggested that these transporters act as "vacuum cleaners" of drugs embedded in the membrane. Nonetheless, how (and whether) these transporters attract themselves therapeutic drugs prior to their extrusion is not known. Our objective was therefore to characterize whether another complementary explanation can be suggested to characterize the "vacuum cleaner" effect. We suggest here that the elusive "vacuum cleaner" hypothesis may be simply explained by a theory based on the lateral Brownian movement of drugs in the membrane. Once partitioned, a drug is not static but diffuses laterally in the membrane during its residency time. The residency time being dependent on drug-membrane physico-chemical interactions, the longer the drug residency time, the longer the lateral diffusive path and thus, the higher is the probability for a drug to meet and be extruded by a transporter. Using these basic assumptions we focused our attention on endocytosis, a process known to be consistently increased in MDR cancer cells. Taking into account of endocytosis we have characterized theoretically the drug/membrane physico-chemical interactions in these conditions. As a result a critical relation between the drugs sizes (cf. Lipinski's rule regarding drugs size/MW), the endocytosis kinetics and the Pgp-like transporters number in the plasma membrane triggering drug resistance is deduced. To test this assumption, positive or negative modulation of endocytosis using specific phospholipids (i.e. phosphatidylserine/PS and phosphatidylcholine/PC; Rauch & Farge 2000), has been performed in Dox resistant cancer cells. As a consequence (see Table 1), we show that alteration of endocytosis can either increase (with PS) or decrease (with PC) the resistance to Dox in agreement with the model suggested. As a conclusion, we confirm that MDR may be the result of physico-chemical and biological synergizing factors. Finally, we provide a new theory of drug partitioning in membranes and related to drug size, endocytosis and Pgp plasma membrane surface density.

Table 1 MCF-7 (breast derived cancer cells) resistant to Dox

	Increase in intracellular Dox fluorescence level (%)
Control (0.7 μM)	100
Control (7 μM)	110
PS (0.7 μM)	52
PS (7 μM)	53
PC (0.7 μM)	106
PC (7 μM)	177

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A rapid supercritical route to cell loaded scaffolds for tissue engineering applications

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Supercritical CO_2 processing ($scCO_2$) is a method of fabricating porous polymer scaffolds for tissue engineering applications without organic solvents and high temperatures. When above its critical point (73.8 bar/31.2°C) CO_2 can dissolve into amorphous polymers such as poly(DL-lactic acid) ($P_{DL}LA$) causing them to plasticise, before subsequent decompression causes the formation of gas bubbles that become permanent as the polymer vitrifies. This has enabled thermally labile biomolecules to be entrapped within $P_{DL}LA$ controlled release matrices using a single processing step (Howdle et al 2001). We hypothesised that a similar $scCO_2$ technique could be used to process live mammalian cells into biodegradable $P_{DL}LA$ scaffolds for tissue engineering applications. This would remove the time consuming and frequently inefficient two-step scaffold seeding process required in many cell-based tissue engineering strategies. However, the retention of cell survival after using such a technique could be problematic as $scCO_2$ has been shown to cause rapid death in bacterial cells (Dillow et al 1999). Despite this, the rapid plasticisation and foaming of $P_{DL}LA$ under supercritical conditions could limit the required cell survival time to less than 5 min. Mammalian cells were subject to a range of $scCO_2$ processing conditions and assessed for metabolic activity using the Alamar Blue assay. It was found that primary hepatocytes, meniscal fibrochondrocytes, myoblastic C2C12s and 3T3 fibroblasts could survive after exposure to $scCO_2$ (74 bar, 35°C) on a time and cell type-dependent basis, with survival above 70% for all cell types after a 1-min exposure. With decompression times of 4 min, cell population metabolic activity remained at least 70% of the control population. Based on this survival data, we developed a one-step $scCO_2$ technique for the rapid production of biodegradable $P_{DL}LA$ scaffolds containing live mammalian cells. A live cell suspension was mixed with $P_{DL}LA$ powder and processed in $scCO_2$ (74 bar/35C) for 1 min. Retained functionality of the cells after $scCO_2$ processing was confirmed by measuring the BMP-2 induced alkaline phosphatase activity of the C2C12 cell line (Tare et al 2002). Levels of gene expression in murine C2C12 cells were also assessed by using a microarray to detect 4,418 genes before subsequent statistical analysis. A significance analysis of microarrays test found that after $scCO_2$ processing for one minute, only eight genes were significantly down regulated when compared to untreated cells. This study demonstrates that mammalian cells can both survive and retain important aspects of functionality after a one minute exposure to $scCO_2$. Survival was found to be time and cell type dependent with extended decompression permitting survival of up to 70% after 4 min. The effects of $scCO_2$ upon gene expression in C2C12 cells after a one minute exposure to $scCO_2$ were minimal, with small down-regulations of eight genes indicating subtle changes at mRNA level that do not lead to significant cell death. In summary, $scCO_2$ can be used to process mammalian cells into porous $P_{DL}LA$ scaffolds using a rapid one-step technique, with cell survival and functionality retained.

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A nano-milled formulation approach to improve the oral bioavailability of a low solubility compound

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Nano-milled suspensions have arisen as a potential strategy for the efficient delivery of low solubility compounds (Patravale et al 2004). Such compounds often have low, variable bioavailability and erratic absorption profiles due to dissolution-rate limited absorption. A case study demonstrating the benefit of using a nano-milled suspension, with reduced particle size leading to improved bioavailability, on a discovery compound for in-vivo toxicology studies is presented. Compound X has a MWt of 331, log D 3.8, thermodynamic solubility of 5 μg/ml at pH 2.3–7.2 and high permeability across Caco-2 cells. Toxicological studies in rat using a standard oral suspension gave linearity and adequate exposures at doses up to 300 mg/kg (Table 1). Since myelotoxicity was observed in rats at doses greater than 100 mg/kg, which appears to be AUC driven, a similar study in dog was conducted to ascertain whether this observed myelotoxicity was species specific. Based on the rat data, the same standard suspension approach was used for the dog study at doses up to 180 mg/kg. Unexpectedly, the required free AUC of >3 ug.h/ml, which demonstrated myelotoxicity in the rat, were not attained in the dog (Table 1). Since the compound has good permeability and was shown to have low first pass metabolism, dissolution-rate limited absorption was postulated as the most likely reason for the poor exposures seen in dog. Formulation strategies to address this challenge were evaluated including the use of co-solvents and stabilized amorphous systems. Formulation assessment, in addition to the simplicity of nano-milled suspensions, resulted in the nano-milled formulation approach being selected for in-vivo evaluation. A nano-milled suspension was prepared by milling the

Table 1 Free AUC and particle size of standard and nano-milled suspensions in rat and dog

	Animal	Dose (mg/kg)	Free AUC (μg.h/ml)	Particle size distribution (μm)
Standard suspension	Rat	300	3.47	10–20
Standard suspension	Dog	60	0.73	10–20
Standard suspension	Dog	180	1.34	10–20
Nano-milled suspension	Dog	60	3.08	0.3–1.3

bulk and media under high shear using acid-washed glass beads for 48 h to produce a concentrated stock. This stock was then diluted down to dose at 60 mg/kg. The nano-milled suspension yielded significant improvements in bioavailability averaging a 4-fold increase in free AUC compared to the standard suspension at 60 mg/kg (Table 1). Particle size analysis showed that particle reduction was a determining factor in bioavailability improvement.

Patravale V. B. et al (2004) *J. Pharm. Pharmacol*. **56**: 827–840

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Automated, real-time monitoring of long-term sustained release delivery systems

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We report on the development of a simple-to-operate, adaptable, economical absorption spectrophotometer applicable to the quantification of long-term sustained drug release. The development of sustained drug release methodologies is of growing interest, particularly in terms of increasing the exposure time of a drug to a specific site (Rathbone et al 2003). However, currently, initial screening of suitable release systems can monopolise expensive and valuable equipment for prolonged periods. To facilitate the study of drug release from devices capable of 4–6 week delivery, the development of a dedicated low cost optical monitoring system has been pursued. In the past ten years significant advances in high brightness, short wavelength light emitting diodes (LED) operating from 300 to 700 nm have revolutionised the lighting and display industry, and in turn have been shown to have applications in the life sciences (Nakamura & Fasol 1997). In line with this application a linear absorption spectrometer utilising these light sources has been constructed and through selection of the correct emitting wavelength the system can be tailored to suite the absorption profile of any drug or compound of interest with a chromophore. The light from the LED is "collimated" and the beam split, whereby a proportion of the light is directed onto a monitoring photodiode whilst the remaining light is made to pass through a glass flow cell before being detected by a second photodiode. Differencing electronics are then used and the signal output amplified before being recorded on a low cost commercial datalogger at fixed time points. The optical system has been developed using precision engineered toy building blocks and baseboard and is enclosed in a light tight box. The sample under test is placed in a plugged dissolution vessel containing a suitable volume of dissolution media, stirred at a constant rate. A peristaltic pump continuously forces the release media from the vessel through the enclosed flow cell and back into the dissolution vessel at a fixed rate, permitting in-line processing. As the compound is released into the media, the amount within the flow cell at any time point absorbs a percentage of the light emitted from the LED and the % light transmitted is reduced, thus altering the signal output, which is then related to concentration. In the initial trial experiments the system has been calibrated and reproducible release profiles have been obtained, comparable with those acquired from manual analysis using a UV/visible spectrophotometer. To date two different compounds with peak absorptions in the visible (630 nm) and ultra-violet (365 nm) have been demonstrated to work. This work has been shown to be effective in the long-term measurement of drug release from sustained delivery systems for use in the gastrointestinal tract. In essence, due to the simplicity of the design, multiple measurements can be run in parallel reducing the time needed for reliable repeated results. It is also felt that the instrument may play a role as an initial screening device for new sustained drug delivery methods permitting rapid elimination of unsuitable devices or formulations.

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