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In vitro delivery of anti-breast cancer agents directly via the mammary papilla (nipple)

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ABSTRACT

The objective of this study was to investigate, in vitro, the plausibility of a novel method for delivering a combination of anti-breast cancer agents to the breast via the mammary papilla (nipple). Mammary papillae were prepared from freshly excised strips of porcine sow breasts by blunt dissection. Permeation studies were performed using all glass Franz diffusion cells in both upright and lateral position, with drugs examined individually and in combination. Donor phase was comprised of equimolar PD98059, LY294002 and tamoxifen; 2.54×10^{-4} mol dissolved in 950 μL fish oil (containing ~23% (w/v) eicosapentaenoic acid, EPA), 25 μL DMSO and 25 μL 1,8-cineole. Also, 4 or 10% Cabosil M5P (w/v) was added to thicken the formulation. After 6 h, the papillae were recovered, cleaned, centrifuged and extracted thrice with methanol. Pooled extracts were analysed by reversed-phase HPLC. The significance of the papilla orientation was also investigated. When applied singly and laterally, the amount extracted from the porcine breast tissue for PD98059, LY294002 and tamoxifen were 1.83 ± 0.30, 10.67 ± 1.78 and $0.74 \pm 0.19 \times 10^{-2}$ μmol g^{-1} respectively; applied simultaneously and laterally, 2.03 ± 0.14, 4.86 ± 0.47 and $0.22 \pm 0.04 \times 10^{-2}$ μmol g^{-1} respectively. With 4% Cabosil formulation, amount extracted for PD98059 and LY294002 were 5.71 ± 0.95 and $9.91 \pm 0.92 \times 10^{-2}$ μmol g^{-1} respectively; with 10% formulation, 2.64 ± 0.5 and $3.90 \pm 0.78 \times 10^{-2}$ μmol g^{-1} respectively. Tamoxifen was below its limit of detection in both Cabosil M5P formulations. To conclude, localized passive delivery via the mammary papilla is a plausible non-invasive means of delivering anti-breast cancer drugs directly to the breast, in levels that have previously been shown to markedly inhibit the growth of breast cancer cell lines, in vitro. The amounts deliverable may be influenced by differential interactions with the thickening agent and patient orientation.

1. Introduction

With more than 45,500 people diagnosed with breast cancer each year in the UK alone (Cancer Research UK, 2008), there exists a major and continued need for novel methods to combat the disease. The combination of PD98059 (Alessi et al., 1995), LY294002 (Vlahos et al., 1994; Gharbi et al., 2007) and tamoxifen (Osborne, 1998) has already been proven to possess potent anti-breast cancer activity, partially attributed to the simultaneous blockade of survival pathways. The recent paper by Davison et al. (2008) demonstrated that, when administered simultaneously, the compounds could penetrate skin in amounts that could reduce the growth of the MCF-7 breast cancer cell line to as little as 2.6 ± 0.01% of control. Fish oil was used as base solvent as it adequately solubilised the 3 drugs and it has well-known anti-inflammatory properties due to high levels of $n-3$ eicosapentaenoic acid (EPA) (Gil, 2002). Furthermore, it has been proposed that $n-3$ fatty acids possess anti-breast cancer activity (Schley et al., 2007).

Currently, chemotherapy for breast cancer relies on oral or intravenous administration of drugs. Complex tablet regimens are often given, which include tamoxifen, aromatase inhibitors and fulvestrant. Such doses are subject to wide distribution throughout the body, first pass hepatic metabolism and side effects. Tamoxifen has been linked with numerous serious side effects including primary endometrial cancer, venous thrombosis and secondary cancer in uterine endometrium. The drug has also been shown to be associated with the formation of covalent DNA adducts in rodents (Han and Liehr, 1992) and the development of liver cancer via a genotoxic mechanism (Davies et al., 1997). Despite these and other undesirable effects, tamoxifen remains the drug of choice in the management of estrogen receptor-positive breast cancer. Tamoxifen resistance also remains a big clinical problem. Insights into (ER+) the mechanisms involved in the development of such resistance have shown a role for the epidermal growth factor receptor (EGFR) and downstream growth factor signaling, thus downstream signaling molecules such as Akt and MAPK (Pearson et al., 2001;

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Nicholson et al., 2004.) have become attractive therapeutic targets. Furthermore, the targets of signal transduction inhibitors are present in, and important to the viability of the majority of cells within the body. Therefore, systemic administration of potent downstream EGFR signal transduction inhibitors, such as LY294002 and PD98059, is implausible as it is highly likely to give rise to severe adverse effects, or even fatalities. To successfully use such a combination of potent compounds would demand a *highly localized* delivery technique.

Most breast carcinomas and pre-cancers originate in the lining of the milk ducts or in the lobules, hence such areas represent the major target for any drug delivery system. The function of the mammary papilla is to concentrate the flow of milk from the breast lobules and facilitate transfer to the infant (Love and Barsky, 2004). As a tissue, it is leaky and would be expected to allow the passive, *non-invasive* transfer of fluids and their solutes in either forward or reverse directions in non-lactating subjects with direct connections to the ducts and lobules. Surprisingly, passive delivery of anti-breast cancer agents via the mammary papilla via the ducts to the lobules appears not to have received attention previously.

The objective of this work was to investigate *in vitro* the plausibility of delivering a combination of anti-breast cancer agents to the breast, directly via the mammary papilla. Using standard Franz diffusion cell methodology and porcine breast tissue, the levels of drug delivered were determined.

2. Materials and methods

2.1. Materials

PD98059 and LY294002 were purchased from Promega, Southampton, UK. Boots 'Omega 3 Fish Oil high-strength' capsules (batch 118202, ~33% EPA) were purchased from a local store. Hanks buffered balanced salt solution (HBBSS), Hepes, 1,8-cineole, tamoxifen and myristyltrimethyl ammonium bromide (cetrimide) were all obtained from Sigma–Aldrich Company Ltd., Poole, UK. Cabosil M5P was a gift from Cabot Corporation, Barry, UK. Strips of porcine sow breasts were obtained post mortem from a local abattoir prior to cleansing procedure and transported to the laboratory in iced Hepes modified Hanks buffered balanced salt solution (HHBBS).

2.2. Preparation of porcine mammary papilla

Freshly excised strips of porcine mammary papilla were washed in tepid water and mammary papilla, surrounded by 2 cm × 2 cm of abdominal skin, were excised by blunt dissection as illustrated in Fig. 1. Subcutaneous fatty tissue was also removed by blunt dissection and pieces were maintained in HHBBS until set up in diffusion cells.

2.3. Test formulations

The delivery of PD98059, LY294002 and tamoxifen were evaluated individually and incombination. The test formulations were based upon those used previously (except that tamoxifen was used in place of 4-hydroxytamoxifen) (Davison, 2008) and were comprised of 4 major actives: equimolar PD98059, LY294002 and tamoxifen (2.54×10^{-4} mol) dissolved in 950 μL fish oil (containing ~33% (w/v) EPA). When used in combination, these were added 25 μL DMSO and 25 μL 1,8-cineole. When thickened, 4 or 10% Cabosil (w/v) was added and stirred vigorously until a homogeneous mix was achieved.

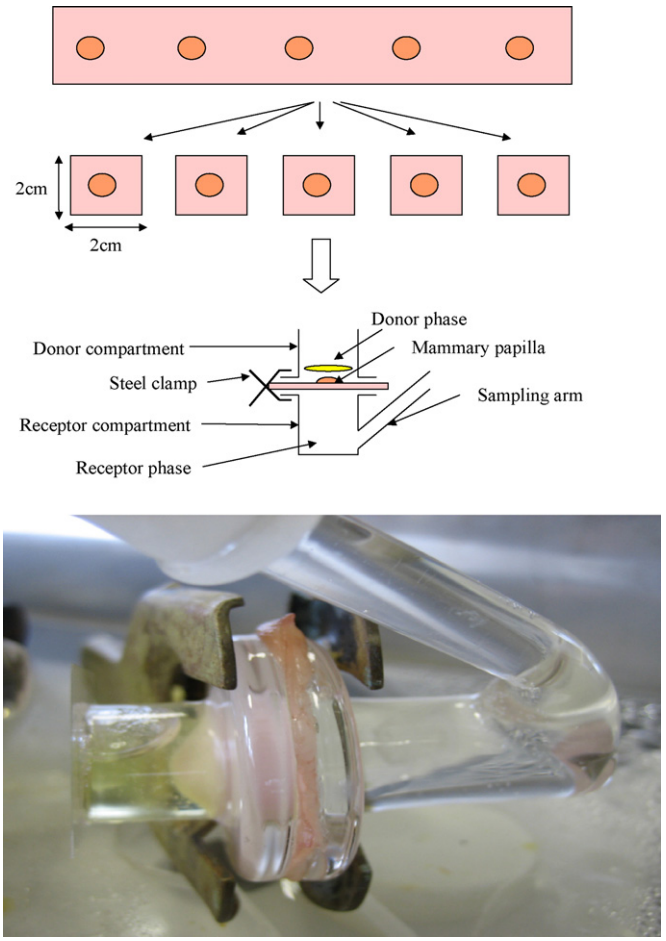

Fig. 1. (Upper) Preparation of sow mammary papilla from breast strips, and mounting in Franz diffusion cells (Davison, 2008). (Lower) Franz diffusion cell set-up showing the porcine mammary papilla *in situ* in lateral position.

2.4. In vitro trans-mammary papilla delivery

The Franz diffusion cell consists of two separate chambers, a donor compartment and a receptor compartment. The porcine breast samples were mounted between the cell compartments, the flanges of which had been smeared with high vacuum silicon grease, with the mammary papilla located in the centre and facing upwards. The two cell compartments were held together with a clamp to minimize leakage. The receptor compartment had a nominal volume of 4.3 mL and was filled with receptor fluid via sampling arm. Micro-stirrer bars were then added and the complete diffusion cells placed on a submersible magnetic stirrer base set up in a water bath at 37 °C. When the breast was mounted in the horizontal position, the donor was sealed with a greased microscope slide and the cell rotated through 90° and supported as necessary (Fig. 1). After 30 min, 500 μL of the drug solution was applied to the surface of the skin by means of a pipette or spatula in the case of the 10% Cabosil formulation. The donor compartment was then occluded with laboratory film. The replication was $n = 4$ and, for consistency with previous work, a receptor solution of degassed cetrimide solution (30 mg mL^{-1}) was used.

2.5. Extraction of drug from breast tissue

After 6 h, the diffusion cells were dismantled and the breast tissue recovered. Excess dose and grease were wiped away and the diffused areas excised and centrifuged at 10,000 × *g* to remove excess solution, cut into approximately 1 mm × 1 mm × 1 mm

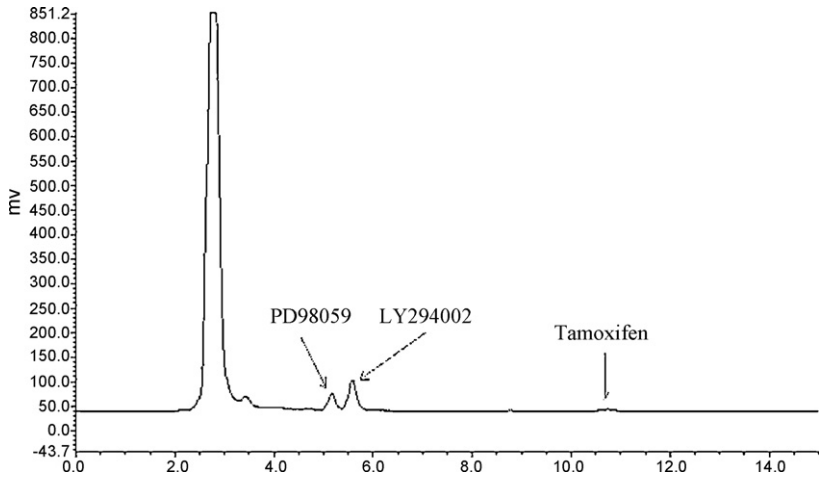

Fig. 2. HPLC chromatogram showing baseline resolution of PD98059, LY294002 and tamoxifen.

cubes with a scalpel then placed into a 5 mL centrifuge tube. Methanol (2 mL) was added and the tube vortex mixed for 30 s before being placed on a rotating blood cell mixer for 30 min. The tubes were then centrifuged at 10,000 × *g* and the supernatant decanted into 10 mL glass bottles. Further aliquots of methanol were added and the extraction process repeated on two more occasions before the pooled supernatants were reduced in a vacuum oven set at 50 °C. The residues were then reconstituted with 1 mL of HPLC mobile phase. This extraction protocol had previously been validated in-house.

2.6. Quantitative analysis

Samples were analysed by reversed-phase liquid chromatography using an Agilent 1100 series automated system with Chemstation software. Analytes were separated using a method developed in-house: Luna C18 ODS 150 mm × 4.6 mm, 5 μm column (Phenomenex, Macclesfield, UK), a gradient elution of 70:30 methanol/water with 0.1% TFA were used to detect the samples, over 15 min at a flow rate of 1.0 mL min^{-1}, 20 μL injection volume, detection was at 254 nm. Fig. 2 shows a representative chromatogram including the 3 analytes using the optimized conditions. Under the conditions used, baseline resolution of PD98059, LY294004 and tamoxifen was obtained, with retention times of 4.5, 6.0 and 10.5 min respectively. Standard calibration curves were constructed over the range of 0.1–20 μg mL^{-1}, which provided linear responses with $R^2 = 1.000$ for each analyte.

2.7. Statistical analysis

Drug delivery into the pappila orientated vertically was compared with that into the papilla orientated laterally by means of a Wilcoxon matched-pairs signed-ranks test, and the combined formulation of PD98059, LY294002 and tamoxifen were compared via a Kruskal–Wallis non-parametric ANOVA test. All statistics were conducted using Instat 3 for Macintosh (Graphpad, CA, USA). Confidence intervals were set at 95% and $p < 0.05$ was deemed as statistically significant.

3. Results

3.1. Comparison of duration of application

Comparing 6 and 48 h, using PD98059 only, the mean mass extracted from the breast tissue were 6.36 ± 0.74 and 7.32 ± 0.84 μg g^{-1} (2.37 ± 0.28 and $2.74 \pm 0.31 \times 10^{-2}$ μmol g^{-1}) respectively. No statistically significant difference between 6 and 48 h permeation tests were found ($p = 0.20$). For this reason, the rest of the experiments were carried out for 6 h only. This also supports earlier findings, where permeation through the mammary papilla was found to reach equilibrium by 6 h (Davison et al., 2008).

3.2. Application of individual drugs to porcine mammary papilla

Table 1 shows the amounts of PD98059, LY294002 and tamoxifen extracted from the excised diffused areas of breast tissue after application of the individual test formulations to the mammary papilla for 6 h. Where delivery was determined in both upright and lateral positions, no significant differences were found ($p > 0.05$). LY294002 has the highest amount extracted from the tissue, followed by PD98059 and tamoxifen, when dosed individually.

3.3. Application of combined formulation to porcine mammary papilla

Table 2 shows the mean amounts of PD98059, LY294002 and tamoxifen obtained from the extracted breast tissue after 6 h permeation. The donor phase was 1 mg of PD98059, LY294002 and tamoxifen in 950 μL fish oil plus 25 μL of DMSO and 25 μL of 1,8-cineole (Davison et al., 2008). Using a Kruskal–Wallis nonparametric ANOVA, the *p*-value for upright and sideway orientation were 0.0036 and 0.0005 respectively. This is considered extremely statistically significant, which means it is very unlikely to have occurred by chance.

3.4. Application of combined formulation thickened with Cabosil M5P to mammary papilla

At a level of 4% Cabosil the formulation remained considerably fluid, while increasing the percentage of Cabosil to 10% provided a substantially more viscous and firm gel formulation, which remained at the point of application i.e. on the top of the nipple rather than tending to flow toward the base; however the nipple was completely covered in each case. Table 2 shows the mean mass of PD98059, LY294002 and tamoxifen obtained from the extracted porcine breast tissue after 6 h permeation (lateral) with a donor phase of the combined formulations with either 4 or 10% Cabosil as thickener. Figs. 3–5 show histograms of drug permeation after 12 h, and highlight the main differences in terms of breast individual drug dosing, combined formulation and thickened combined formulation respectively.

Table 1
Amounts of PD98059, LY294002 and tamoxifen extracted from breast tissue in the upright and lateral positions individually after 6 h ($n = 4$, ±SD).

	Amount extracted after 6 h				*p*-Value
	Upright[a]		Lateral[a]		
	$\mu g\,g^{-1}$	$\mu mol\,g^{-1}$	$\mu g\,g^{-1}$	$\mu mol\,g^{-1}$	
Dosed individually					
PD98059	6.36 ± 0.74	$2.37 \pm 0.28 \times 10^{-2}$	4.89 ± 0.81	$1.83 \pm 0.30 \times 10^{-2}$	0.026
LY294002	53.69 ± 17.46	$17.47 \pm 5.68 \times 10^{-2}$	32.79 ± 5.47	$10.67 \pm 1.78 \times 10^{-2}$	0.026
Tamoxifen	0.73 ± 0.08	$0.20 \pm 0.01 \times 10^{-2}$	2.06 ± 0.31	$0.55 \pm 0.04 \times 10^{-2}$	>0.05
Dosed as a combined formulation					
PD98059	18.24 ± 6.96	$6.82 \pm 2.60 \times 10^{-2}$	5.44 ± 0.36	$2.03 \pm 0.14 \times 10^{-2}$	>0.05
LY294002	45.62 ± 12.27	$13.32 \pm 1.40 \times 10^{-2}$	14.94 ± 1.47	$4.86 \pm 0.47 \times 10^{-2}$	>0.05
Tamoxifen	6.93 ± 6.28	$1.86 \pm 1.69 \times 10^{-2}$	0.81 ± 0.15	$0.22 \pm 0.04 \times 10^{-2}$	>0.05

[a] Diffusion cell orientation.

Table 2
Amounts of PD98059, LY294002 and tamoxifen extracted from lateral breast tissue in combined formulations containing either 4 or 10% Cabosil after 6 h ($n = 4$, ±SD).

	Amount extracted after 6 h				*p*-Value
	4% Cabosil		10% Cabosil		
	$\mu g\,g^{-1}$	$\mu mol\,g^{-1}$	$\mu g\,g^{-1}$	$\mu mol\,g^{-1}$	
PD98059	15.26 ± 2.55	$5.71 \pm 0.95 \times 10^{-2}$	7.05 ± 1.35	$2.64 \pm 0.50 \times 10^{-2}$	0.0286
LY294002	30.46 ± 2.84	$9.91 \pm 0.92 \times 10^{-2}$	11.98 ± 2.40	$3.90 \pm 0.78 \times 10^{-2}$	0.0286
Tamoxifen	<LOD	<LOD	<LOD	<LOD	–

LOD, (analytical) limit of detection.

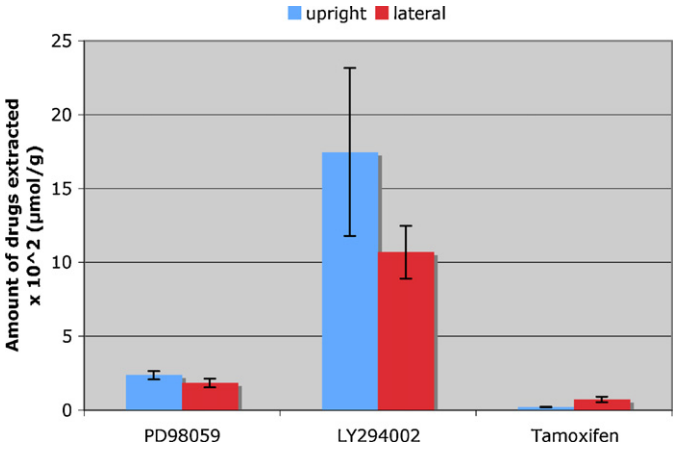

Fig. 3. The amount of PD98059, LY294002 and tamoxifen extracted from breast tissues in the upright and lateral permeation after 6 h, when dosed individually.

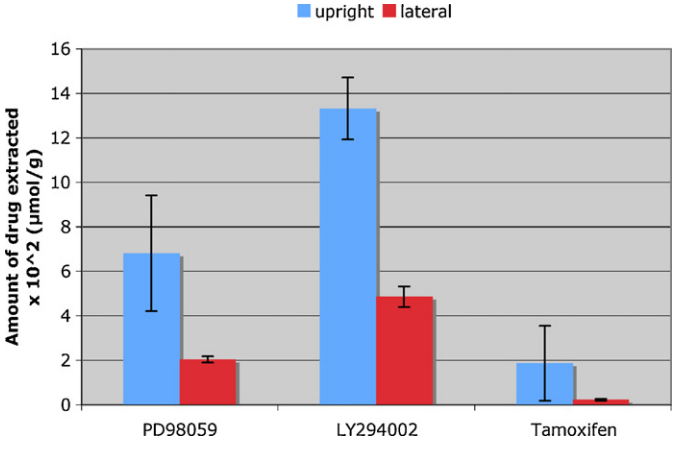

Fig. 4. The amount of PD98059, LY294002 and tamoxifen extracted from breast tissues in the upright and lateral permeation after 6 h, when dosed with combined formulation.

4. Discussion

Current breast cancer chemotherapeutics are predominantly delivered through the systemic route orally or by i.v. injection. This method has been relied upon for many years and only recently that the idea of localized delivery (i.e. ductoscopy and intraductal injections) has received attention. Although systemic administration is the most efficient route of delivery to cancers in many organs, it also exposes all the healthy tissues to the delivered drugs, resulting in harmful side effects. For example, endometrial cancer is a severe side effect of tamoxifen that can, at least partially, be attributed to systemic dosing. It is estimated that 95% of breast cancers arise from the epithelial cells of the gland lobules and ductal network of the breast (Murata et al., 2006). The main hypothesis in this work suggests if an effective localized delivery system could be developed that only targeted such tissues, it could limit or circumvent entirely such side effects.

Until recently direct access to this area has been by core biopsy or fine needle aspiration only (Shen et al., 2000) although the newer techniques of 'ductography' and 'fiberoptic ductoscopy' have been

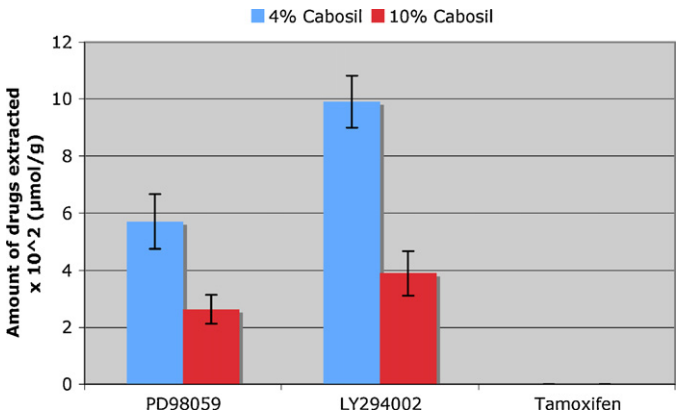

Fig. 5. The amount of PD98059, LY294002 and tamoxifen extracted from breast tissues in the lateral permeation after 6 h, when dosed with combined formulation containing either 4% or 10% Cabosil M5P.

explored and proposed for visualisation in the diagnosis of breast cancer and other diseases of the breast (Yamamoto and Tanaka, 2004). Ductoscopy has also been investigated for delivering breast cancer treatment to the tumour directly. Although deemed 'minimally invasive' this procedure was painful to the extent that general anaesthesia was necessary. The passive diffusion of chemical dyes throughout the full ductal networks has been mapped previously, although these were applied via intraductal injection (Murata et al., 2006). Intraductal injection also provides direct access to breast lesions with higher local and lower systemic drug exposure (Murata et al., 2006). However, in practice, such injections are likely to be at least discomforting for the patient. A passive delivery system, whereby the active agents are released from an applied formulation and diffuse through the conduits of the papilla would be virtually pain-free and likely to be far more attractive to a patient.

The leakiness of the mammary papilla, hence potential as a portal for drug delivery of anti-breast cancer agents, was recently demonstrated in vitro (Davison, 2008), using an excised sow breast/Franz diffusion cell model. The mass transport of these agents was demonstrated through the conduits of the papilla, although the amounts of compound localized within the tissue had not been determined. One of the main aims of this work was to obtain tissue concentrations of the active compounds using the same experimental set-up, as used previously. The previous work employed Franz diffusion cells containing a receptor phase of 30 mg mL^{-1} cetrimide as a sink. Although sink conditions were not necessarily required in the current work, the same receptor phase was employed for consistency. There are conflicting views on whether this solution compromises the integrity of barriers; although *any* receptor phase has the potential to migrate towards the donor phase when the delivery mechanism involves some form of microconduit. From the very low levels of drug extracted (below), coupled with no visible sign of 'reverse' migration of receptor fluid, it can be suggested that the use of this receptor phase was not detrimental to the data obtained.

The use of excised porcine mammary papilla involves a number of assumptions. To fit the diffusion cell, the breast tissue needed to be trimmed substantially even though anatomically, the pig breast is more compact than the average human. The integrity of the lobules following blunt dissection was checked by microscopic examination following staining with 0.1% (w/v) methylene blue in Sorenson's buffer (0.1 M).

It was previously found that the migration of drugs through the papilla reached equilibrium after 6 h, it was assumed that a similar timescale was appropriate in the current work (Davison, 2008). A brief experiment supported this, where donor phase containing PD98059 was left for 48 h and was found to deliver the same amount of compound as that found after 6 h.

In considering the end-user of a trans-papilla drug delivery device, it is apparent that the delivery process should be examined in two orientations: (1) with the papilla facing upwards (mimicking the patient in a *recumbent* position) and (2) with the papilla in a lateral orientation (mimicking the patient in an *upright* position). To examine potential differences, diffusion cells were set up in both normal and lateral orientation. For all three compounds, there was no significant difference between the two positions at the 5% level ($p > 0.05$). However, with p-values very close to 0.05 in each case (0.06, 0.06 and 0.10 for LY294002, PD98059 and tamoxifen respectively) it is reasonable to suggest that the delivery mechanism is somewhat dependent on other forces, such as gravity. Thus the amounts of compound delivered may be affected by everyday activities such as standing and sleeping.

In the non-thickened combined formulation, the differences between the two permeations (upright and lateral) are not significant (p 0.10) for each of the PD98059, LY294002 and tamoxifen. By comparing individual compounds permeate across the tissue, LY294002 showed highest permeation level followed by PD98059 and tamoxifen, when dosed individually and in combined formulation. This can be explained by tamoxifen and PD98059 having higher binding affinity towards the breast tissues when compared to LY294002. However, it was previously shown that after 5 h 24.69 ± 3.31, 17.87 ± 2.44, 20.13 ± 2.89 $\mu g\,cm^{-2}$ of PD98059, LY294002 and 4-hydroxytamoxifen passed into the receptor phase respectively. In the current work the amount of LY294002 extracted from the combination was the highest, unlike in the work of Davison (2008) where LY294002 permeation was found to be the lowest. This seems rational in that if LY294002 is preferentially absorbed by the tissue then less will be available to permeate.

Increasing the Cabosil content from 4% to 10% (w/v) resulted in substantially decreased masses of PD98059 and LY294002 by 54% and 61% respectively. This is readily explained in the closer association or binding of compound to the thickening agent (Gallagher et al., 2003). The 10% (w/v) Cabosil produced a much more viscous and firm gel formulation, hence the drug release rate is decreased and the drug tends to remain on the skin layer rather than penetrate through the skin. This also supports the earlier findings, where the lag time was increased and the time taken to reach equilibrium was delayed with the permeation of increased Cabosil percentage (Davison, 2008). Tamoxifen was not detected in the receptor phase following the administration of formulations thickened with Cabosil. This could be explained by the fact that tamoxifen is metabolized into 4-hydroxytamoxifen in the presence of cytochrome P450 isoform CYP2D6 (Desta et al., 2004). However, no unidentified peaks were observed in the HPLC analysis. This could therefore suggest that tamoxifen was preferentially binding to the silica particles of the Cabosil thickener, although Davison (2008) observed migration of tamoxifen through the papilla from both of the thickened formulations. This then points to a third potential explanation involving competitive binding within the breast tissue, where PD98059 and LY294002 occupy binding sites which are then unavailable for molecules of tamoxifen.

Overall, the levels of active extracted from the mammary papilla tissues were very low. When approximately 2×10^{-4} mol of each drugs were dosed in combined formulation on the mammary papilla, laterally, 2.03 ± 0.14, 4.86 ± 0.47 and $0.22 \pm 0.04 \times 10^{-2}$ $\mu mol\,g^{-1}$ of PD98059, LY294002 and tamoxifen were extracted from the tissues respectively. In the current work, it is not possible to extrapolate such numbers to human breast, although the amounts would be expected to be greater in the normal female breast, but possibly comparable in the average male breast. To determine whether the amounts delivered represent potentially efficacious doses, it would normally be appropriate to compare with IC_{50} data. Similar and moderate inhibition of MCF-7 breast cancer cells was observed previously when dosed with PD98059 and LY294002 individually (Davison et al., 2008). However, when used in a combined formulation, they showed substantially greater inhibition towards MCF-7 growth (Davison et al., 2008). The IC_{50} concentrations of tamoxifen, PD98059 and LY294002 had already been determined in-house on MCF-7 and TamR cells as 1×10^{-7}, 25×10^{-6} and 5×10^{-6} M respectively (Davison et al., 2008). However, the IC_{50} of the combination of all 3 agents could not be determined as growth was effectively eliminated. This was accounted for in that they all work by blocking the different pathways involved in cell signaling and lead to the notion that a 'cocktail' therapeutic system is more beneficial towards breast cancer. The apparent absence (i.e. <limit of detection) of tamoxifen binding within the papilla tissue may appear to be at odds with the last statement. However, another important finding by Davison et al. (2008) was that the combination of PD98059 and LY294002 was as effective in eliminating the growth of MCF-7 cells as was the mixture containing tamoxifen; it was suggested that the inclusion of tamoxifen may not be necessary. It

can therefore be proposed that, based on the amounts of PD98059 and LY294002 delivered alone, a potentially therapeutic system is plausible, based upon this work.

5. Conclusions

This study has confirmed that the actives of the formulation used in this experiment may be delivered successfully via the mammary papilla. The localized delivery of anti-cancer agents directly via this route is a simple, non-invasive and potentially effective way of circumventing the considerable issues concerned with other forms of delivery of anti-breast cancer agents. In particular, the high doses and wide distribution of these potent agents can be reduced by using the trans-mammary delivery system, which required lower doses and locally distributed hence minimal side effects.

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