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Research Paper

In Vitro Effects on MCF-7 Breast Cancer Cells Of Signal Transduction Inhibitor/Tamoxifen/Eicosapentaenoic Acid Combinations and their Simultaneous Delivery Across Skin

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Purpose. To determine the *in vitro* effects of simultaneously administered LY29400, PD98059, tamoxifen and eicosapentaenoic acid (EPA) on breast cancer cells, and determine their transcutaneous delivery.
Methods. Growth assays were performed on MCF-7 cells challenged with IC_{50} and permeated concentrations of PD98059, LY294002 and tamoxifen firstly in isolation then combined. Permeation studies were performed using PD98059 and LY294002 (singly or simultaneously) in DMSO then fish oil, with enhancers. Immunocytochemical detection of phospho-MAPK, phospho-Akt, total COX-2 and Ki-67 was performed.
Results. When applied singly, fluxes of PD98059 and LY294002 were 0.09 ± 0.008 and 0.14 ± 0.045 μg cm^{-2} h^{-1}, respectively; applied simultaneously, 0.18 ± 0.045 and 0.49 ± 0.051 μg cm^{-2} h^{-1}. Permeated concentrations of PD98059 and LY294002 reduced growth to $13.78 \pm 0.63\%$. Fish oil plus 2.5% DMSO/ethanol allowed 5.96 ± 0.9 and 7.7 ± 1.2 μg cm^{-2} of PD98059 and LY294002 to permeate after 48 h.
Conclusions. PD98059 and LY294002 permeate excised skin at therapeutically useful rates, and also demonstrate growth inhibitory effects on MCF-7 cancer cells. Synergism was noted in co-transport across skin and activity against cancer cells. A formulation based on fish oil is potentially skin friendly; simultaneous permeation of EPA provides further anti-cancer action.

KEY WORDS: breast cancer; EPA; signal transduction inhibitor; tamoxifen; transcutaneous delivery.

INTRODUCTION

Breast cancer is currently the most common cancer in the UK with around 41,000 new cases diagnosed each year. In the western world, an estimated one in nine women develop breast cancer at some stage in their life (1). Tamoxifen, a selective estrogen receptor modulator, remains the gold standard treatment for hormone sensitive, estrogen receptor positive (ER+) breast cancer. Intrinsic resistance affects around 30% of patients who do not respond to tamoxifen treatment. Acquired resistance is also thought to affect many initially responding patients, which is thought to lead to the development of a more aggressive phenotype (2, 3).

Unchallenged, ER + breast cancer cells normally proliferate through ER signalling and so initially, when this pathway is challenged with tamoxifen, cancer cell growth is inhibited. However, after prolonged challenge the cancer cells acquire resistance to tamoxifen by adopting a new route of growth and proliferation. In particular, increased epidermal growth factor receptor (EGFR) signaling is commonly seen both clinically and in breast cancer cell lines that have developed endocrine resistance (4). Two downstream mediators of the EGFR, mitogen-activated protein kinase (MAPK) and phosphoinositol-3 kinase (PI3K) have been implicated as being involved in hormone resistant growth. Their activation can promote growth factor signalling, inhibition of apoptosis and cell survival, and proliferation. It is now also understood that activation of PI3K and MAPK can lead to ligand-independent activation of the ER, giving rise to ER activation irrespective of tamoxifen binding. Activated MAPK and PI3K can phosphorylate key serine residues (118 and 167, respectively) of ER, which leads to its activation, also promoting pro-cell survival events (5–8). Therefore, these two mediators are attractive targets in order to achieve inhibition of hormone resistant growth. It is hypothesised that tamoxifen resistance could be delayed or sensitivity could be restored by inhibiting these pathways.

Akt is a direct target of PI3K and is activated in a PI3k-dependant way by growth factors. Its activation has been shown to lead to phosphorylation of cell survival proteins such as caspase-9 and so is crucial to cell survival (9) LY294002 is a selective PI3K inhibitor, developed from the naturally occurring bioflavanoid, quercetin. It acts by competitively inhibiting the ATP binding site of PI3K, therefore preventing the transfer of the terminal phosphate of ATP to phosphoinositol (10). Activation of the MAPK pathway leads

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ABBREVIATIONS: EGFR, epidermal growth factor receptor; EPA, eicosapentaenoic acid; ER, estrogen receptor; k_p, permeation coefficient; 4OHTam, 4-hydroxytamoxifen.

to the transcription of many tumour-promoting proteins such as jun and Elk-1 (11). PD98059 is a potent and selective inhibitor of MAP kinase kinase (also known as MAPK/ERK kinase or MEK kinase). Inhibition of this pathway would decrease the production of these proteins and so is a plausible way of treating the anti-estrogen resistant phenotype. The compound PD98059 prevents phosphorylation and so activation of MAPK or ERK1/2, without acting directly on MAPK (12).

Using a combination therapy comprised of tamoxifen, LY294002 and PD98059 it is postulated that tamoxifen resistance could be largely delayed or even eradicated. However, the targets of both signal transduction inhibitors are present in, and important to the viability of the majority of cells within the body, so systemic administration of these compounds is highly likely give rise to severe or maybe even fatal adverse effects. However, the breast represents a reasonably accessible target for topical administration, where the drugs can be delivered transcutaneously using a semi-solid formulation or device, and which then undergo passive diffusion to the underlying tissues (13). The primary advantage of such *local* targeting would be much reduced quantities of drug circulating in the blood stream (and non-target tissues); other benefits include the elimination of first-pass hepatic metabolism (although first pass dermal metabolism is possible), controllable zero-order rates of delivery, and potential for the rapid termination of dose in event of adverse reaction (14). Furthermore, topical delivery systems are generally well-accepted and tolerated by patients, thus aiding compliance.

Deriving a topical formulation for LY294002 and PD98059 must take account of their lipophilicity (clogP 3.03 and 2.57, respectively) and oil bases are frequently used in such cases. Some oils possess intrinsic anti-cancer properties by virtue of their high content of bioactive polyunsaturated fatty acids. It is known that many breast tumours over express COX-2 and have elevated levels of PGE_2 (15, 16). A study of COX-2 expression with molecular, clinical and pathologic markers involved in breast cancer concluded that strong COX-2 staining was significantly correlated with high tumour stage and BRE grade and that there was a strong correlation with metastatis, suggesting that COX-2 may be involved in breast cancer progression and cell motility (17). A further study involving selective COX-2 inhibitors demonstrated dose dependant decreases in cell proliferation and migration in two breast cancer cell lines, in addition to dose-dependant inhibition of metalloproteinases MMP 2 and MMP 9, (18) which have been implicated in motility and metastasis (19). Eicosapentanenoic acid (EPA) competes with arachadonic acid (AA) for the enzymatic site of the COX-2, leading to the development of low potency prostanoids, compared to the high potency prostanoids made via utilisation of AA. Fish oil is comprised of up to 60% EPA and also has been shown to knockdown COX-2 expression during skin penetration (20). Thus there exists a rationale for the use of fish oil, which could serve not only as a lipophilic base for LY294002 and PD98059, but also possesses intrinsic anti-cancer activity.

This work was thus concerned with the development of a novel transcutaneous therapeutic system for breast cancer, involving the simultaneous administration of LY294002, PD98059, tamoxifen and EPA. Fig. 1 shows a flow chart representation of proposed effects of this combination of drugs.

MATERIALS AND METHODS

Materials

PD98059 and LY294002 were purchased from Promega, Southampton, UK. Fish oil capsules (Boots super-strength, ~40% EPA) were purchased from a local store. Hanks balanced buffered salt solution (HBBSS), HEPEs, gentamycin sulphate, sodium bicarbonate PBS, PBS+0.02% Tween, sodium citrate, citric acid, methyl green, copper sulphate, paraffin wax pellets, 30% hydrogen peroxide solution, sodium hydroxide, hydrogen chloride, DPX mountant, 1,8-cineole, 4-hydroxy tamoxifen and myristyltrimethyl ammonium bromide (cetrimide) were all obtained from Sigma-Aldrich Company Ltd, Poole, UK. Phospho-MAPK primary antibody, phospho-Akt and COX-2 primary antibodies were from Cell Signalling Technology, New England Biolab (Hitchin, UK). HRP labelled anti-rabbit polymer, normal goat serum, DAB chromagen plus substrate, Ki-67 primary antibody and delimiting pen were from Dako, Ely, UK. Ethanol, xylene, Whatman filter papers (45 μm), Whatman parafilm, high

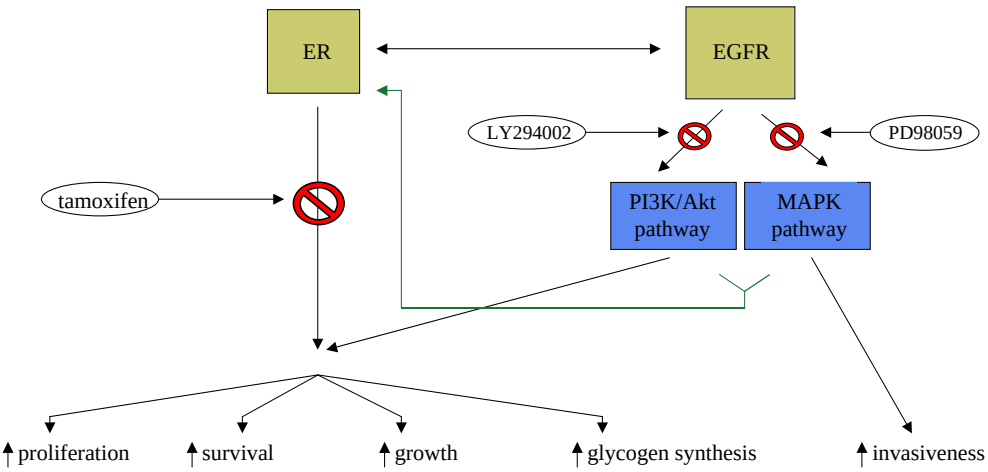

Fig. 1. Flow diagram representing the pathways involved in breast cancer growth, proliferation and survival (derived in house).

vacuum silicon grease and chloroform were from Fisher Scientific (Loughborough, UK). All cell culture reagents and FCS were from Invitrogen Life Technologies (Paisley, UK). Tissue culture plasticware was from Nalge Nunc International (Roskilde, Denmark). Parental MCF-7 cells were a gift from AstraZeneca, Macclesfield, UK. Porcine ears were obtained from a local abattoir prior to steam cleaning.

Cell Culture

MCF-7 cells are an adherent epithelial breast cancer cell line, derived from a pleural effusion adenocarcinoma. Cells were routinely cultured in RPMI-1640, which was supplemented with 5% FCS, 10 U ml^{-1} and 10 μg ml^{-1} penicillin/streptomycin and 2.5 μg ml^{-1} fungizone. Media was changed every 4 days and cells were passaged weekly by trypinization. Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air.

To determine the effect of the active compounds on cell growth MCF-7 cells were grown to ~60–70% confluency in a T-75 cm^2 sterile plastic flask, trypsinised and set up in phenol-free wRPMI, supplemented with 5% charcoal-stripped FCS, 10 U ml^{-1} and 10 μg ml^{-1} penicillin/streptomycin and 2.5 μg ml^{-1} fungizone at 1.5 million cells per plate in 24 well plates. The cells were left overnight to attach to the wells. After this 24 h equilibration, base counts were taken and then cells were treated with pre-determined IC_{50} concentrations of PD98059 (25×10^{-6} M), LY29002 (5×10^{-6} M) or 4-hydroxy tamoxifen (10^{-7} M), 1×10^{-7} M estradiol to act as a positive control (to ensure cells were estrogen responsive and a model for ER+ve breast cancer) or negative control (0.025% *v/v* DMSO in media). Cells were counted a further three times between days 2 and 9 using a Coulter Multisizer II counter and re-treated with media and treatments on day 4. Media was removed on re-treatment, which mimicked dermal clearance to a certain extent. These studies were repeated with combinations of 25×10^{-6} M PD98059 plus 5×10^{-6} M LY294002 and 10^{-7} M tamoxifen, 25×10^{-6} M PD98059 plus 5×10^{-6} M LY294002 and were compared to the single treatments. Passage numbers were 18, 20, 22.

The effect of the permeated concentration of EPA was assessed on the growth of MCF-7 cells. Cells were dosed with 4 μl of fish oil per 1 ml of media to deliver the accurate permeated amount of EPA to the cells on days 1 and 4. Fish oil was dissolved 1:1 with ethanol. Counts were taken on days 1, 3, 5 and 8. Passage numbers were 14, 15, 17.

Lastly, growth curves were also determined using concentrations of PD98059 and LY294002 found to have permeated skin after 48 h. Concentrations were taken from the *in vitro* permeation studies (0.75×10^{-6} and 1.5 10^{-6} M, respectively). The studies were conducted as above, except that cells were treated 0.75×10^{-6} M PD98059 and 1.5 10^{-6} M LY294002 on day 1 and then with the concentration permeated after 24 h, each day thereafter (0.38×10^{-6} M PD98059 and 0.75 10^{-6} M LY294002). Passage numbers were 21, 23, 25.

Preparation of Skin Membranes

Porcine ears, obtained immediately post slaughter from a local abattoir, were immersed in iced Hanks balanced buffered salt solution (HHBBSS). The dorsal skin was liberated from underlying cartilage by blunt dissection to provide sheets of approximately 0.8 mm in thickness, whilst being continually bathed in HHBBSS, before being cut into 2 cm^2 sections and used immediately.

In Vitro Transcutaneous Delivery

Porcine ear skin is increasingly being viewed as a valid model for human skin (22). Transcutaneous delivery, across whole skin to underlying tissues, was modelled using glass Franz-type diffusion cells (nominal diffusional area 0.8 cm^2, nominal receptor volume 3.5 ml), in which the skin membranes were mounted stratum corneum uppermost between the greased flanges. The diffusion cells were assembled with the stratum corneum uppermost and held together with stainless steel clamps. The receptor phase was degassed aqueous cetrimide 30 mg ml^{-1}—an effective solubiliser of such lipophilic compounds (Table I) which does not compromise the integrity of the skin membrane (13,23) Magnetic stirrer bars were added and the complete cells were placed on a VarioMag Telesystem submersible stirrer plate (Camlab LTD, Cambridge, UK) set up in a Clifton unstirred water bath (Nickel Electro LTD, Weston-super-Mare, UK) and maintained at 37°C to provide a surface temperature of 32°C via heat dissipation. After 30 min equilibration time the cells were dosed with 200 μl of PD98059 or LY294002 in DMSO or fish oil, previously prepared to provide equimolar aliquots of 2.5×10^{-4} M (approx 2×10^{-4} mol cm^{-2}). Although unsuitable clinically, DMSO was used as vehicle in order to determine maximal permeation. The sampling arms of the receptor phases were capped and the donor phases were occluded with Whatman Parafilm. At 3, 6, 12, 24 and 48 h the entire contents of the receptor phases were removed and replenished with fresh solution. Samples, 1 ml, were stored at −20°C in autosampler vials prior to analysis.

HPLC Analysis

Samples were analysed by reverse phase liquid chromatography using an Agilent 1100 series automated system with Chemstation software. Samples were separated using a method developed in-house: Luna C18 ODS 150×4.6 mm, 5 μm column (Phenomenex, Macclesfield, UK), a gradient elution of 80:20 to 70:30 methanol/water over 20 min at a flow rate of 0.5 ml min^{-1}, 20 μl injection volume, detection was at 254 nm. Under these conditions baseline resolution of PD98059 and LY294004 was obtained, with retention times

Table I Solubility of PD98059 and LY294002 in Cetrimide Solution (Receptor Phase), DMSO and Fish Oil (Vehicles) (n=3, ±SD)

Vehicle	Cetrimide, mg ml^{-1}	DMSO, mg ml^{-1}	Fish oil, mg ml^{-1}
PD98059	4.57±0.76	6.98±0.34	2.47±0.13
LY294002	9.42±0.98	14.76±1.08	5.16±0.41

of 12.5 and 14.5 min, respectively. Standard solutions (prepared in receptor phase) provided linear responses with r^2=1.000. The samples containing a fish oil vehicle were analysed separately for EPA. Again, samples were separated on a Phenomenex Luna C18 column (150×4.6 mm, 5 μm), however an isocratic elution of 20:80 methanol/water with 2.5% acetic acid was employed and detection was at 210 nm. EPA, under these conditions, had a retention time of 6.5 min. The amounts permeated were determined against the appropriate standard calibration curves (also prepared in receptor phase), which provided linear responses with R^2=1.000 for each analyte. Cumulative permeation μg cm^{-2} were plotted against time and the linear portion of the resulting plot taken as steady state flux.

Statistical Analysis

Welch corrected, unpaired t tests were carried out using Instat 3 for Macintosh (GraphPad software, USA).

Immunocytochemical Analysis of Phospho-MAPK, Phospho-Akt and Total COX-2 in Porcine Skin

Skin membranes were mounted in Franz diffusion cells as described above, except a receptor phase of HHBBSS was used to maintain skin viability, then dosed with PD98059 plus LY294002 in DMSO or fish oil, fish oil alone or water. At 0, 3, 6, 10, 24 and 48 h, membranes were removed and the exposed areas excised before being cut into approx 5×2 mm pieces. These were then fixed in 4% formaldehyde and dehydrated through a series of increasing concentration ethanol baths and xylene baths, after which, the skin was embedded in wax. The embedded skin was sectioned using a Leica manual rotary microtome and sections were straightened in a RA Lamb section water bath set at 37 °C and then transferred onto 3×1 inch pre-cleaned microslides. Sectioned skin was assayed for the detection of Ki-67, phospho-Akt, phospho-MAPK and total COX-2 using protocols described in the primary antibody pack leaflets.

RESULTS

MCF-7 Growth Studies Using IC_{50} Concentrations

To serve as a positive control, the effect of all three compounds, individually and in combination, on MCF-7 cell growth was investigated firstly using their respective IC_{50} values. When incubated individually with MCF-7 cells, all three compounds were found to inhibit growth, as shown in Fig. 2 (left). Cell growth was reduced to 59.4±5.8% of control with 4-hydroxytamoxifen treatment, 68.4±3.8% with PD98059 treatment and 54.9±2.3% with LY294002 treatment after 8 days. Although from this figure it would appear as if LY294002 had the greatest inhibitory effect on cell growth, cell growth continued to increase linearly at day 8 with LY294002 treatment, whereas growth with tamoxifen and PD98059 reached a plateau between days 6 and 8.

Fig. 2 (right) shows the effect of combinations of tamoxifen, PD98059 and LY29002 when incubated with MCF-7 cells. It is apparent that the mixture of PD98059 plus LY29002 and the combination of all three compounds dramatically inhibited cell growth. It is also apparent from this figure that there was no significant difference whether 4-hydroxytamoxifen was included in the mixture or not. The combination of the two signal transduction inhibitors reduced cell growth to 2.8±0.02% of control, whereas the cocktail of PD98059, LY294002 and 4-hydroxy tamoxifen reduced it to 2.6±0.009% of control (p>0.05). For this reason 4-hydroxy tamoxifen was not taken forward to the permeation studies.

Permeation of PD98059 and LY294002 from DMSO Vehicle

DMSO was used as vehicle in initial experiments as it is widely known as a potent penetration enhancer (10), thereby representing the maximal permeation achievable. Typical zero-order permeation profiles were obtained using both the single and simultaneous permeation studies of PD98059 and LY294002, with steady state flux taken between 12 and 48 h. Table II shows the flux for PD98059 and LY294002 were 0.09

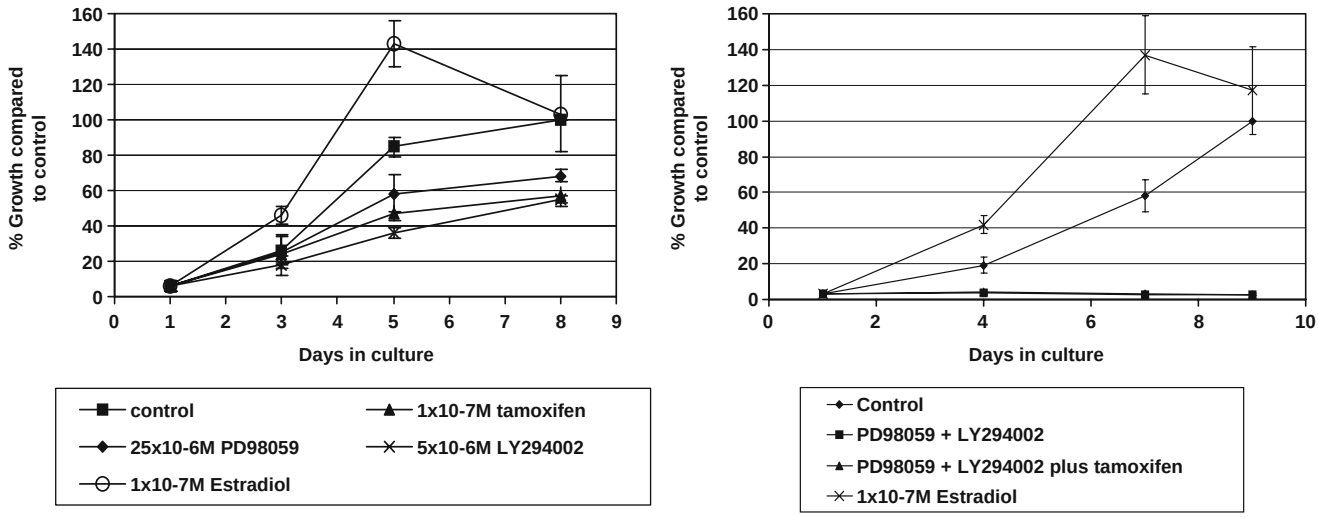

Fig. 2. Growth curves to show the effects of single (*left*) and cocktail treatments (*right*) of control (0.025% DMSO), 4-hydroxy tamoxifen, PD98059, LY294002 and the effects of estradiol were studied to ensure that the cells were estrogen responsive on MCF-7 growth over 8 days. Passage numbers 18, 20, 22 (n=3 ±SD).

Table II Steady State flux (J_{ss}), Cumulative Permeation at 24 and 48 h (Q_{24} and Q_{48}) and Permeability Coefficient (k_p) Data For the Delivery of PD98059 and LY294002 Across Excised Full Thickness Porcine Skin When Applied Singly or Simultaneously (n=6, ±SD)

	Dosed singly PD98059	LY294002	Dosed simultaneously PD98059	LY294002
J_{ss}, μg cm^{-2} h^{-1}	0.09±0.008	0.14±0.015	0.18±0.045	0.49±0.051
Q_{24}, μg cm^{-2}	0.9±0.03	1.46±0.04	4.59±0.88	6.28±1.04
Q_{48}, μg cm^{-2}	3.3±0.43	4.9±0.65	9.0±1.77	17.95±1.30
Q_{24}, μmol cm^{-2}	0.003±0.0006	0.0047±0.0006	0.017±0.004	0.02±0.003
Q_{48}, μmol cm^{-2}	0.01±0.002	0.02±0.003	0.034±0.008	0.058±0.09
k_p, cm h^{-1}	4.5×10^{-4}	7.0×10^{-4}	1.25×10^{-3}	2.99×10^{-3}

±0.008 and 0.14±0.045 μg cm^{-2} h^{-1}, respectively. The fluxes were significantly different (p 0.0438) despite equimolar amounts of each being dosed to the skin. The k_p values are 4.5×10^{-4} and 7×10^{-4} cm h^{-1} for PD98059 and LY294002, respectively, clearly showing the greater intrinsic skin permeability of LY294002. This is reflected in cumulative permeation data at both Q_{24} and Q_{48}.

When dosed simultaneously, there were large increases in the permeation of both compounds. For PD98059 the flux doubled to 0.18±0.045 μg cm^{-2} h^{-1} (p<0.0001); for LY294002 almost quadrupled to 0.49±0.051 μg cm^{-2} h^{-1} (p<0.0001). Similarly, these data were reflected in the cumulative permeation data. The k_p data are also an order magnitude greater, indicating a penetration enhancing effect.

The Effect of Permeated Concentrations of PD98059 and LY294002 on MCF-7 Growth

With the concentrations of PD98059 and LY24002 permeated known, it was important to assess the effects of these amounts on MCF-7 cell growth. Based upon a device of 25 cm^2 in area, it would be possible to deliver 0.75×10^{-6} M PD98059 plus 1.5×10^{-6} M LY294002 through the skin. Fig. 3 shows that when MCF-7 cells were treated with the permeated concentrations of PD98059 and LY294002, in a patch application model system, MCF-7 growth was reduced to 66.2±5.99%, 61.5±5.17%, 13.78±0.63% of control, when treated with PD98059, LY294002 and a combination of both PD98059 plus LY294002. Clearly, the combination is acting synergistically in inhibiting cell growth.

The Effect of DMSO and Fish Oil on Skin Viability

Although the data demonstrate the plausibility of delivering PD98059 and LY294002 across skin, the use of high amounts of DMSO would not be acceptable in practice. To verify this, skin treated with DMSO was assessed for viability by both haematoxylin staining and by immunocytochemical detection of Ki-67, which is a proliferation marker and detects actively proliferating cells. As the skin is a continuously proliferating organ, this assay is a suitable way of determining viability. The haematoxylin staining clearly showed that DMSO, compared to skin dosed with water, caused delamination of the stratum corneum and keratinocyte nuclei loss. The Ki-67 assay showed a loss in the proliferation marker after only 3 h of treatment with DMSO (Fig. 7B) when compared to skin assayed at time 0 h (Fig. 7A). It also demonstrated stratum corneum thinning and keratinocyte toxicity, again demonstrating that the skin quickly lost viability when compared to 0 h control skin section. However, skin treated in a similar fashion using fish oil demonstrated sustained viability after 24 h, as shown by a Ki-67 assay, this is shown in Fig. 7C. Here it is apparent that stratum corneum thickness and histology closely resembles

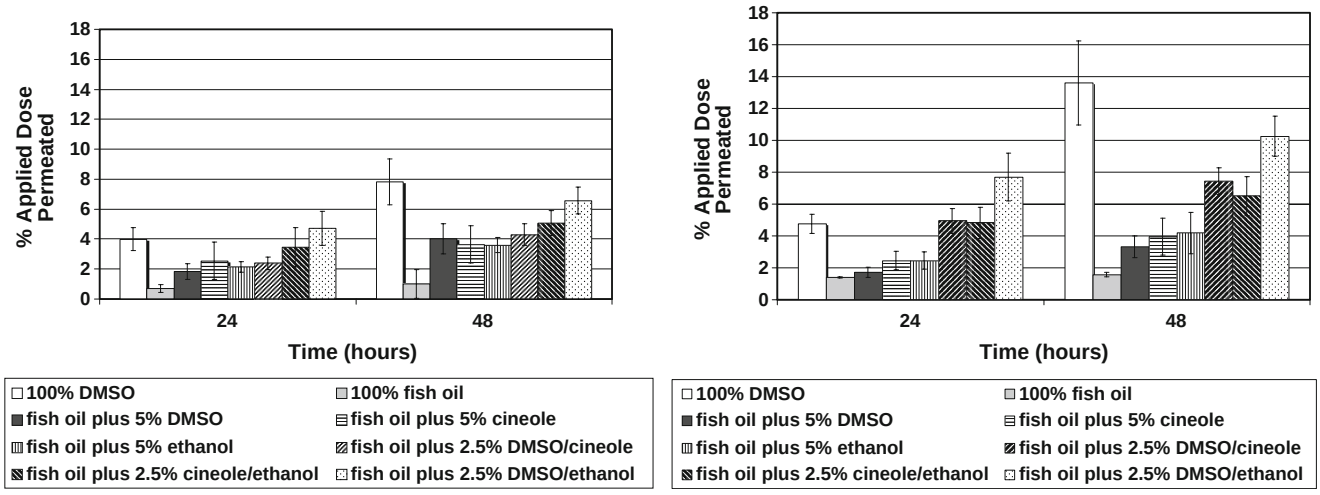

Fig. 3. Histograms to show the percentage of dose applied of PD98059 (*left*) and LY294002 (*right*) permeated after 24 and 48 h with different formulations. Compounds were applied simultaneously (n=5 ±SD).

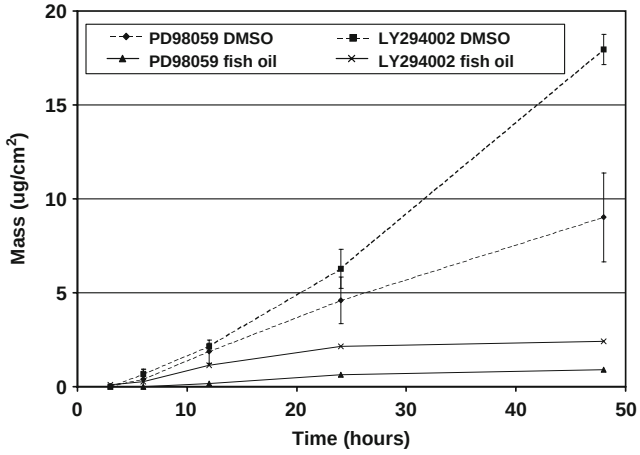

Fig. 4. Permeation profiles showing the difference in permeation of PD98059 and LY294002 from both a DMSO vehicle and a fish oil vehicle.

that of the 0 h control (Fig. 7A) and Ki-67 staining is detected at similar quantities, suggesting that fish oil maintains the viability of the keratinocytes and the skin tissue as a whole.

Permeation of PD98059 and LY294002 from Fish Oil Vehicle

The permeation of PD98059 and LY294002 was determined from a fish oil vehicle, based on its low deleterious effects on skin and intrinsic anti-cancer activity of EPA, if delivered successfully. Fig. 4 shows that the permeation of both PD98059 and LY294002 are, as expected, considerably lower compared to DMSO. The permeation profiles show that 1.51±0.07 and 2.67±0.06 μg cm^{-2} of PD98059 and LY294002 permeated excised porcine skin, respectively, after 48 h. When compared to the mass permeated from the DMSO vehicle, it can be seen that only around a tenth of both PD98059 and LY294002 permeated from a fish oil vehicle. Steady state fluxes of PD98059 and LY294002 were 0.04±0.013 and 0.083±0.05 μg cm^{-2} h^{-1}, respectively.

Permeation of PD98059 and LY294002 from Fish Oil Vehicle Containing Enhancer

Considering the lower fluxes from fish oil relative to DMSO, the effect of incorporation of chemical enhancers was investigated. Firstly, the effect of 5% DMSO, 5% ethanol or 5% 1,8 cineole in fish oil vehicle was examined. Secondly the effect of combining enhancers (2.5% DMSO/1,8-cineole, 2.5% 1,8-cineole/ethanol and 2.5% DMSO/ethanol) was explored. Fig. 5 (left) shows that all three enhancers produced approximately fourfold increases relative to fish oil alone in the permeation of PD98059 (approximately half that observed with 100% DMSO). The enhancers facilitated PD98059 permeation to 3.63±1.1, 3.49±1.2, 3.28±0.5, 3.9±0.68, 4.59±0.7 and 5.96±0.9 μg cm^{-2}, respectively, for fish oil plus 5% DMSO, ethanol and 1,8-cineole and 2.5% DMSO/1,8-cineole, 1,8-cineole/ethanol and DMSO/ethanol at 48 h.

Fig. 5 (right) shows the effect of the three enhancers on the permeation of LY294002 in a fish oil base. The histogram shows that the three enhancers and enhancer combinations had less of an effect on the permeation of LY294002 than PD98059. The addition of 2.5% DMSO/ethanol seemed to have the greatest enhancing effect after 48 h, but still only increased permeation to a half of that seen with 100% DMSO. The addition of the enhancers facilitated 5.07±1.1, 6.68±2.01 and 6.4±1.96, 5.4±1.2, 4.85±1.1, 7.7±1.2 μg cm^{-2} for 5% DMSO, ethanol and 1,8-cineole and 2.5% DMSO/1,8-cineole, 1,8-cineole/ethanol and DMSO/ethanol, respectively, at 48 h.

Overall, Fig. 5 suggests that the combination of 5% DMSO and ethanol provided the greatest permeation enhancement of PD98059 and LY294002. Small amounts of DMSO and ethanol can generally be tolerated in topical formulations.

The permeation of EPA from a fish oil vehicle was also calculated, as shown in Fig. 6 (left). The permeation profile for EPA shows that 266.95±31.18 μg cm^{-2} permeated through excised skin after 48 h. Thus the three anti-cancer actives were demonstrated to successfully permeate the skin.

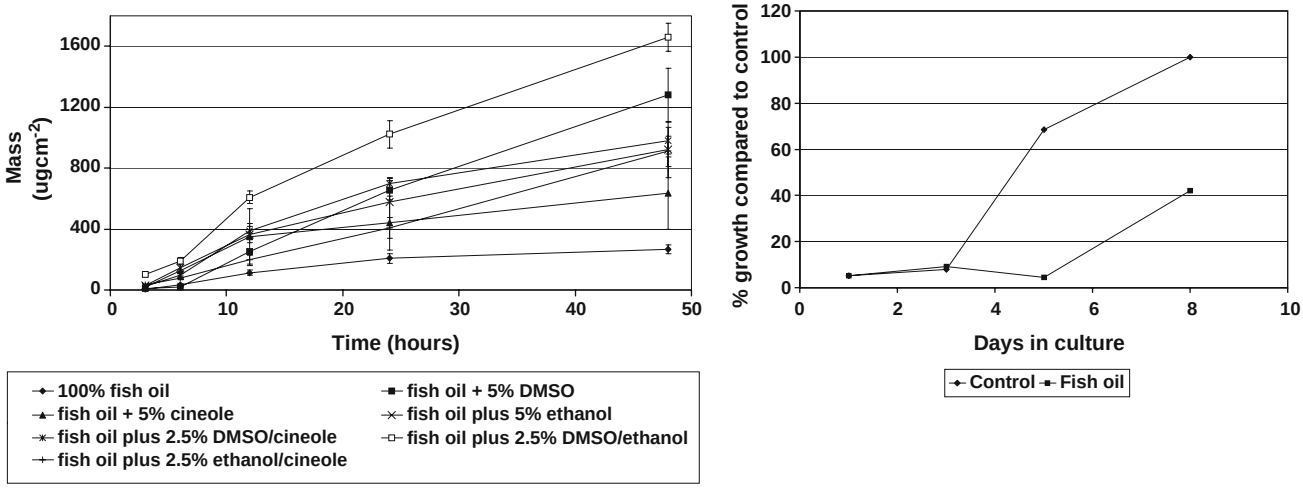

Fig. 5. Left: Permeation profile to show the mass of EPA permeating excised porcine skin after 48 h with different formulations (n=5 ±SD). Right: Growth studies to show the effect of permeated concentrations of EPA on MCF-7 breast cancer cells. Control is 0.025% *v*/*v* ethanol. Passage numbers 14, 15, 17 (n=3 ±SD).

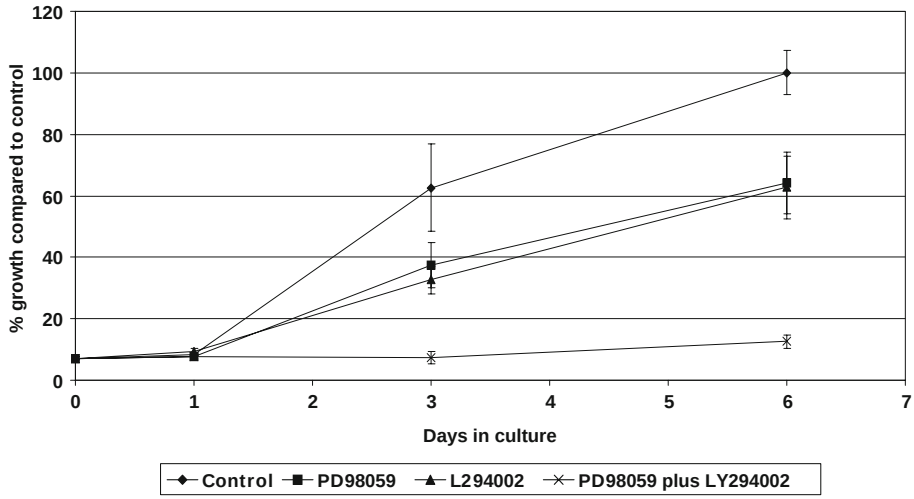

Fig. 6. Growth curve to show the effect of permeated concentrations of treatments of PD98059 and LY294002 on MCF-7 cell growth. Cells were treated with permeated concentrations of PD98059 and LY294002 after 48 h on day 1 (0.75×10^{-6} and $1.5\ 10^{-6}$ M, PD98059 and LY29002, respectively) and then with the concentration permeated after 24 h, each day thereafter (0.38×10^{-6} M PD98059 and $0.75\ 10^{-6}$ M LY294002). Control is 0.025% *v*/*v* ethanol. Passage numbers 21, 23, 25.

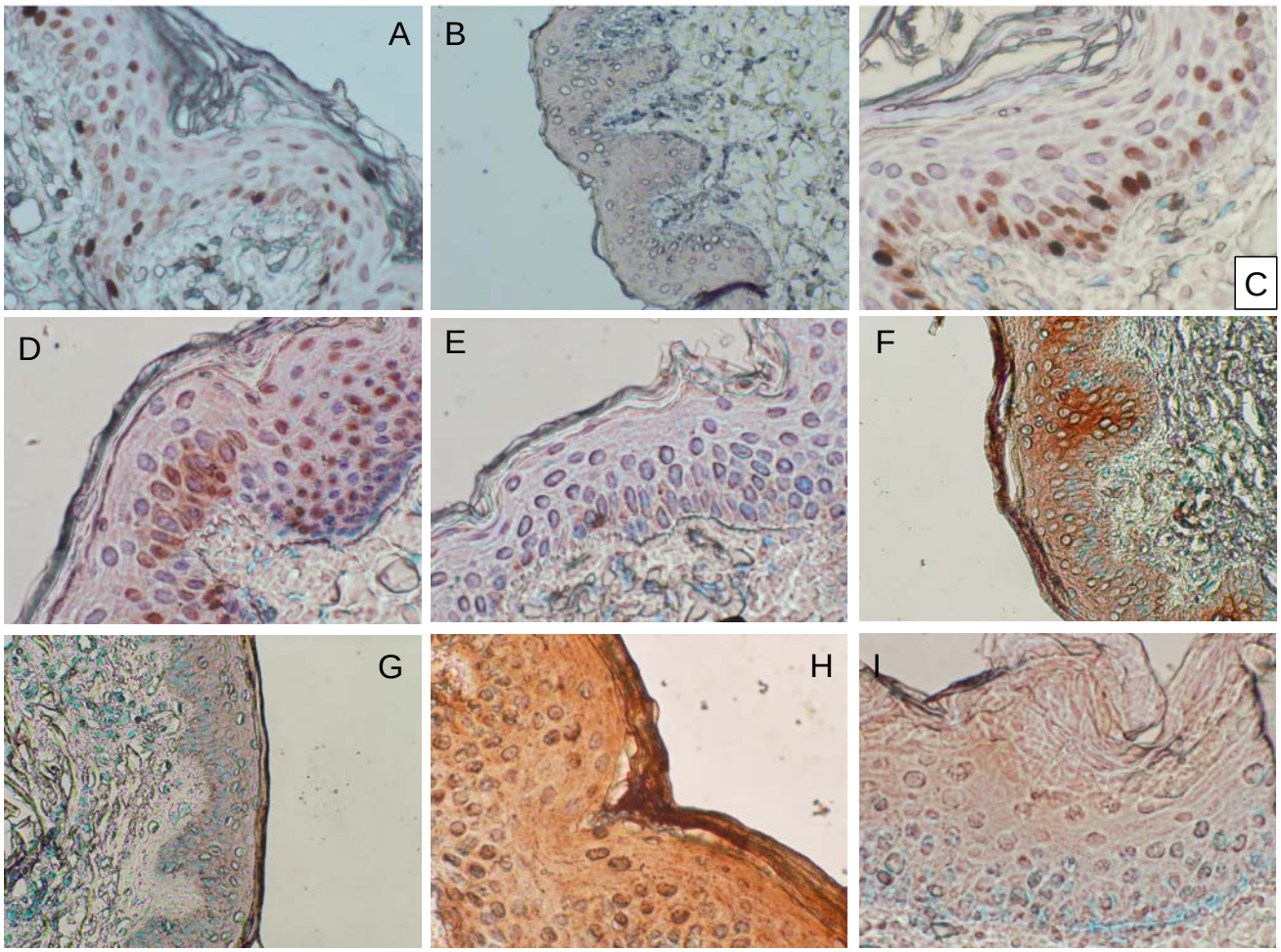

Fig. 7. Sections of porcine skin (5 μm thick) stained for total Ki-67 (**A–C**) with **A** representing 0 h control, **B** is 3 h incubation with 200 μl DMSO and **C** being skin incubated with 200 μl fish oil. P-MAPK (**D**, **E**) p-Akt (**F**, **G**) and total COX-2 (**H**, **I**) after 6 h incubation with either test formulation (**E** PD98059 in fish oil, **G** LY294002 in fish oil and **I** fish oil) or control formulation (**D** and **F** fish oil, **H** water) Concentrations were 200 μl of 1 mg ml^{-1}. The receptor phase was HHBBSS and was stirred with a micro stirrer. Incubation was at 37°C. The skin was removed and processed after 6 h (n=3).

The Effect of Fish Oil on MCF-7 Cell Growth

The concentration of EPA found to have permeated the skin was determined and the appropriate amount of fish oil, dissolved in ethanol and applied to cells on days 1 and 4. Fig. 6 (right) shows that permeated concentrations of fish oil (alone) dramatically reduces MCF-7 growth. After 7 days in culture and in the presence of fish oil, MCF-7 growth was reduced to 44.1±4.56% of ethanol control.

Immunocytochemical Analysis of the Effect of the Formulation on p-MAPK, p-Akt and COX-2 in the Skin

This work was performed to provide confirmatory evidence that PD98059, LY294002 and EPA retained activity during the permeation process, and to predict activity beyond the skin after permeation and diffusion into the underlying tissues. The target of PD98059 is MEK, which when inhibited stops the phosphorylation and thus activation of MAPK. Fig. 7 shows the effect of topically applied PD98059 on p-MAPK in porcine skin, indicating that the activated MAPK was localised within the epidermis and predominantly displays nuclear staining, which is most abundant at the basal layers of the epidermis (Fig. 7D). Cytoplasmic staining is also seen, which is again more concentrated at the basal layers. Fig. 7E clearly shows that even after 6 h there is a marked decrease in activated MAPK within the epidermis of the PD98059 treated skin compared to fish oil treated control (Fig. 7A). This pattern continues up to 24 h, where, after this stage the skin lost viability and the control skin only displayed low levels of activated MAPK (data not shown).

Fig. 7 also shows the effect of LY294002 treatment on activated Akt levels. LY294002 is a potent inhibitor of PI3K, which lies upstream of Akt in this pathway. Therefore p-Akt staining was assessed to show the effect of LY294002. It can be seen that p-Akt staining is mainly cytoplasmic (Fig. 7F), and that after 6 h staining is greatly reduced (Fig. 7G).

The expression of COX-2 is induced by a range of stimuli, including tissue damage and inflammation. The process of excision was sufficient to induce an inflammatory response in the skin (20) and so COX-2 would be induced and would be active prior to use. The skin had been pre-treated with either water, to act as control or fish oil alone. At time 0 (data not shown) COX-2 was present equally in both water control and fish oil treated skin, suggesting that excision from the pig and further dissection within the lab elicited an inflammatory response, allowing knockdown in total COX-2 levels to be more easily observed. Fig. 7 shows that the COX-2 enzyme is localised to the epidermis, supporting the knowledge that this is the enzymatically and metabolically active constituent of skin. At 6 h, however, it can be clearly seen that application of fish oil markedly reduces total COX-2 levels (Fig. 7H) compared to water treated skin (Fig. 7I), suggesting that the fish oil was capable of entering the skin and inhibiting COX-2.

DISCUSSION

MCF-7 cell growth was reduced by a similar margin with the addition of all three compounds being investigated. ER signalling does not solely control cancer cell growth, EGFR signalling contributes to cancer cell proliferation, growth, survival and invasion to differing degrees depending on the cell phenotype. For instance, it has been shown that in the absence of estrogen EGF displays a much more potent proliferative effect and that cancer cells are more likely to use this ligand to promote their growth and it has been suggested that this would lead to elevated expression of EGFR (21). Also, MCF-7 cells that have been exposed to anti-estrogen challenge subsequently express elevated levels of EGFR and develop anti-estrogen resistance (3).

The three compounds inhibited MCF-7 growth to similar degrees when applied individually. This trend was expected as these compounds block different pathways involved in cell signalling, all of which are similarly important to growth and proliferation of the MCF-7 cells (Fig. 1). When each of the pathways was blocked, the MCF-7 cells were able to use an alternative pathway in which to grow and survive. It has previously been demonstrated that significant inhibition of MCF-7 cell growth could be achieved with incubation of either 5 μM LY294002 or 15 μM PD98059 (22). It has also been shown that the concentrations used in this research were enough to inhibit MCF-7X cells (estrogen and growth factor depleted) by 65% and 35% with LY294002 and PD98059 treatment, respectively (24). Greatest inhibition was seen with the 'cocktail' treatments. When combined 4-hydroxytamoxifen, PD98059 and LY294002 reduced cell growth to 2.6% of control treated cells and there was some evidence of cell death. It was also observed the synergistic actions of PD98059 and LY294002, reporting that this combination reduced cell growth to 75% (24). The three signalling pathways that are blocked by these compounds represent three major, although not sole, routes that cancer cells can employ to promote their growth and survival and so sequential blockade of these pathways should lead to cessation of cell growth. However, a similar result was seen with just the signal transduction inhibitors in the absence of 4-hydroxytamoxifen, with MCF-7 cell growth reduce to 2.8%. Cell growth was not expected to decrease by the same amount when 4-hydroxytamoxifen was absent from the cocktail as the ER signalling pathway was left unchallenged. It was expected that MCF-7 cells would still grow and proliferate, to some degree, via this pathway. This may mean that the MAP kinase and PI3 kinase pathway are so crucial to cell proliferation and viability that blockade of these pathways is enough to prevent cell growth, and even bring about cell death.

Fig. 1 shows the targets of all three compounds. Breast cancer cells have the potential to adopt either the ER or EGFR pathway to grow and proliferate. Activated PI3K and MAPK can activate ER in a ligand-independent manner. When all three compounds were given together the MCF-7 cells may have been proliferating mainly via the EGFR pathway, without a significant amount of ligand independent activation of ER occurring. Thus the inclusion of 4-hydroxytamoxifen did not improve the growth inhibitory effects of PD98059 and LY294002, and was omitted from the formulation.

In transcutaneous delivery the aim is to deliver drugs across the whole skin to underlying regions. However, a certain amount of clearance by the microvasculature is inevitable, which could actually be beneficial. The permeation rates of the two compounds increased when applied together compared to when they were applied individually. This could

be a consequence of the increased thermodynamic activity when both compounds were present in the vehicle; alternatively, it could have been due to a complexation effect (25,26). Sub-saturated solutions were used on grounds of the availability of PD98059 and LY294002 and the fact that the simultaneous permeation of vehicle, in particular fish oil, renders thermodynamic activity considerations of little value. From fish oil, both signal transduction inhibitors (and EPA) permeated the full thickness porcine skin and the reduced amounts of permeated PD98059 and LY294002 relative to DMSO could be due damage caused by DMSO to the stratum corneum and/or lower solubility in fish oil by approximately two thirds. Under the conditions used the fluxes of PD98059 and LY294002 and EPA were zero-order, demonstrating the plausibility of a sustained release transcutaneous delivery system. The delivery of EPA also proves the permeation of the vehicle.

It is not straightforward to compare the amounts of drug delivered across the skin to the amounts used in the cell growth experiments. On the face of it, the 25×10^{-6} M of compound used to stop the cell proliferation does not compare favourably with the maximum delivery of 0.06 μmol cm^{-2} after 48 h—an area of application ~400 cm^2 would be necessary. Even then this does not take into consideration the precise location within breast tissue of the cancer to be reached via passive diffusion (currently under investigation) or the proportion of drug cleared by the dermal microvasculature (although the amount in the systemic circulation would probably be beneficial). Such considerations point to the chemoprevention of breast cancer a more plausible clinical application for transcutaneous delivery of these agents, rather than intervention therapy.

However, permeated concentrations were applied to MCF-7 cells in a repeat dosing model, where concentrations permeated after 48 h were first applied and the concentrations permeated after 24 h were applied every 24 h thereafter. Although the concentrations that were added were 33.3 and 3.3 times lower than previously used in the IC_{50} experiments, cell growth was still reduced to 13.78±0.63% of control when both compounds were added together. Although this may appear surprising, a possible explanation is the different uptake rates of the compounds into MCF-7 cells. The IC_{50} value concentrations were only dosed twice during the week-long experiment, whereas the permeated concentrations were dosed on a daily basis. If the uptake of compounds into cells was less or around equal to the concentrations dosed per day in the permeated concentrations investigations then increasing the concentration would not improve the growth inhibition seen. However, it has been reported previously through Western blot techniques that 100 nM PD98059 was adequate to block MAPK significantly (27). This concentration is lower than the 0.75 μM used in these experiments, which suggests that the inhibition was specific to MAPK inhibition.

CONCLUSIONS

Using excised porcine skin as a model, this work has indicated that PD98059 and LY294002 permeated breast skin in amounts that also demonstrated growth inhibitory effects on MCF-7 cancer cells *in vitro*. Synergy was noted in the co-transport of PD98059 and LY294002 across skin AND activity against cancer cells. A formulation based on fish oil was found to be skin-friendly, and the simultaneous permeation of EPA provides a further anti-cancer action. As proof of concept, the data support the development of a multi-pronged transcutaneous system, probably best suited to chemoprevention of breast cancer.

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