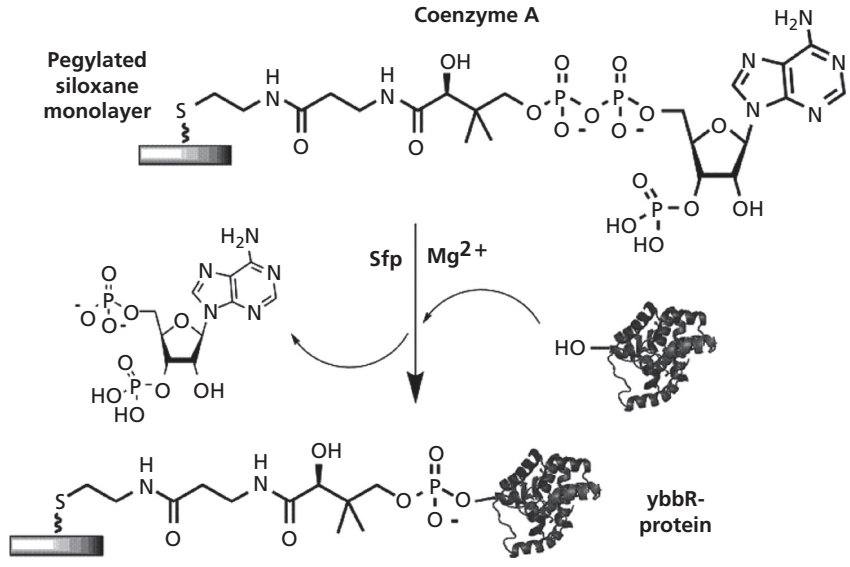

Figure 1 General scheme of the attachment of proteins to immobilised CoA.

relatively small number of proteins are immobilised on nanoscale features. Here, the phosphopantetheinyl transferase Sfp was used to site specifically and covalently immobilise proteins bearing the small ybbR-tag[1] onto nanopatterned siloxane monolayer surfaces bearing coenzyme A (CoA).

Method

The gene for a model protein thioredoxin (Trx) was produced as a ybbR-Trx fusion protein using standard molecular biology techniques. Bioresistant photoreactive siloxane monolayers were prepared and patterned by exposure to 325-nm laser light.[2] CoA was subsequently attached to the exposed areas *via* a suitable maleimide linker molecule. The ybbR-protein was immobilised by attachment to the surface CoA under Sfp as catalyst (Figure 1). The surfaces were then imaged by atomic force microscopy (AFM).

Results and Discussion

The AFM images show an increase in height in the patterned areas corresponding to the immobilisation of the ybbR-Trx. This was further confirmed by binding with anti-Trx antibodies, which gave a further increase in height. In contrast, in control experiments where the catalysing enzyme Sfp was omitted, no height increase was observed, confirming that Sfp was required for immobilisation. In areas that were not previously exposed to light, no proteins were bound, indicating bioresistance was maintained. Using scanning near-field photolithography, submicro patterns as small as 150–200 nm could be obtained.

Conclusion

The use of Sfp-mediated protein immobilisation onto a nanofabricated siloxane surface is demonstrated. This mild, facile and covalent method is site-specific in terms of the site of attachment on the protein, as well as with respect to the location on the nanofabricated monolayers. This is thus a significant step towards the development of a variety of highly miniaturised biomedical instruments.

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Short Papers in Pharmaceutical Science

24
In-silico identification of the skin sensitisation potential of active pharmaceutical ingredients

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Introduction and Objectives

The aim of this study was to assess the potential of an in-silico method to identify the skin sensitisation potential of active pharmaceutical ingredients. Recent efforts have been focussed on the identification of the skin sensitisation potential of (nonpharmaceutical) substances (e.g. for the Registration, Evaluation Authorization, and restriction of

Chemicals (REACH) legislation). These approaches have been centred around the hypothesis that skin sensitisation is brought about through the covalent interaction of toxicant and skin immunoprotein (i.e. reactivity). The chemical mechanisms associated with skin sensitisation have been defined, but not in the context of active pharmaceutical ingredients.

Method

Information on the skin sensitisation potential of active pharmaceutical ingredients was retrieved from publicly available safety data sheets supplied by pharmaceutical companies on their corporate Internet sites. For substances for which the potential of skin sensitisation was identified, the information was summarised as either sensitiser or nonsensitiser. All compounds were coded as 2-D SMILES strings and entered into an in-house algorithm that encodes the mechanistic chemistry defined by Enoch *et al.*[1] to identify protein-reactive substances. The performance of the in-silico approach was assessed by comparison with the known data and a contingency table created.

Results

The skin sensitisation potential of 107 active pharmaceutical ingredients was retrieved from corporate Internet resources. Of these, 74 active pharmaceutical ingredients were associated with skin sensitisation. While the rules for protein reactivity were not trained on pharmaceutically active substances, they are able to identify the majority (47 compounds, 64%) of skin sensitisers. Of all the compounds that were recognised as containing an alert for protein reactivity, a high proportion (78%) were skin sensitisers. The analysis indicates that the coding of mechanistic chemistry is significantly associated with identifying the skin sensitisation potential of active pharmaceutical ingredients. The misclassified compounds provide useful information to develop these rules further to handle issues such as metabolism and to extend the domain of the models. This provides a basis to extend the coverage of the rules for protein reactivity associated with skin sensitisation. Further, analysis of the structure of compounds that are identified *in silico* as having the potential for skin sensitisation, but for which there is no evidence, will provide information on structural mitigating factors such as deactivating groups.

Conclusion

The results show that useful toxicological information may be extracted from safety data sheets, which will assist in the development of in-silico toxicological approaches. The approach applied here was developed previously for nonpharmaceutical compounds and can be extended to active pharmaceutical ingredients. Such in-silico approaches will provide a means to identify the skin sensitisation properties of new pharmaceutical substances, reducing the requirement for costly animal tests.

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25
Development of FK506-binding protein like and its peptide derivatives as antiangiogenics and antimetastatic therapeutics for the treatment of cancer

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Introduction and Objectives

It has been recognised that dysregulated angiogenesis is key for switching dormant tumours to invasive and metastasising malignancies. Approved antiangiogenics targeting the vascular endothelial growth factor (VEGF) such as Avastin show limited potency and problematic drug resistance.[1] These agents can promote tumour invasiveness.[2] We have evidence to suggest that FK506-binding protein like (FKBPL) is an antiangiogenic protein, which targets the cell surface receptor, CD44, rather than the VEGF pathway. CD44 has well-characterised roles in cellular migration, adhesion, survival and invasion, i.e. the key steps involved in angiogenic and metastatic processes.[3]

Method

We have designed potent therapeutic peptides based on the antiangiogenic domain of FKBPL with a view to develop them as novel therapeutics.[4,5] Since many of the same processes involved in angiogenesis are also important for tumour metastases, this study investigates the antimetastatic potential of these peptides at a cellular level to explore the impact on tumour cell migration, adhesion and invasion. The molecular mechanisms through which the peptide exerts its antimetastatic activity were also explored.

Results and Discussion

We showed that these peptides actively delayed the migration of malignant cells without showing any cytotoxic activity. The peptide-mediated inhibition of migration was likely due to the increased adhesive capacity of the cells since we showed a peptide-mediated increase in adhesion to the CD44 extracellular ligand, hyaluronan. Using a microarray approach, we were able to identify a subset of genes, such as RhoA, vinculin and profilin, which were regulated by the peptide. These genes are associated with actin binding and the formation of focal adhesions key to

migratory and adhesive processes. These changes in gene expression were confirmed at the protein level using Western blot analysis.

Conclusions

We hypothesise that FKBPL and its peptide derivates are potent antimetastatic as well and antiangiogenic agents that mediate their activity by modulating CD44 pathways associated with cytoskeletal dynamics promoting cell adhesion and inhibiting migration.

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26
A new approach to the treatment of liver cirrhosis: transglutaminase inhibitors and potential delivery systems

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Introduction and Objectives

Transglutaminase (TG) inhibitors can be used in the treatment of TG relevant human diseases including chronic neurodegeneration, neoplastic diseases, autoimmune diseases and fibrosis.[1] In our previous work, we quantify the relative efficacy and toxicity *in vitro* of different transglutaminase inhibitors.[2] This work investigates the biodistribution and potential application of fluorescent TG inhibitors and future application of TG inhibitors-entrapped liposomes in the treatment of liver cirrhosis.

Method

For biodistribution studies, Wistar rats were injected with 50 mg/kg of 10-mg/ml TG inhibitor intraperitoneally. After predetermined time intervals, blood samples and organs such as liver, lungs, kidneys and brain were removed for further analysis. To extract the TG inhibitor from the organs, 250 mg of each organ was homogenised in methanol and centrifuged. The supernatant was collected and read by fluorimetric estimation using high-performance liquid chromatography. The half-life of the drug and the amount of distributed drug in each organ was determined. The activity of the extracted drug in organs tested was investigated by an enzyme-linked sorbent assay.

Results and Discussion

According to the results, the half-life of the drug was very short (20 min). This could be due to hydrophobicity of the drug. Moreover, it passed through tissue membrane and was found to be widely distributed within all organs tested. The drug was shown to remain active after extraction from the tested organs.

Conclusion

Considering the in-vivo clearance of liposomes by the liver, incorporation of TG inhibitors inside liposomes could be an appropriate approach for reducing the large drug distribution, eliminating the associated side effects and passively targeting the liver, with the aim of treating liver cirrhosis. Further results using liposomal carriers in this respect will be reported.

Acknowledgements

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27
Modified Franz diffusion for the in-vitro determination of the efficacy of antimicrobial wafers against methicillin-resistant Staphylococcus aureus

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Introduction and Objectives

Antimicrobial wafers offer great potential for sustained topical drug delivery directly to a suppurating and infected

wound bed. It is necessary to quantify the efficacy of these lyophilised delivery systems, containing clinical concentrations of broad spectrum antimicrobial compounds, against methicillin-resistant Staphylococcus aureus (MRSA).

Methods

Lyophilised wafers were produced by casting karaya gels containing clinical concentrations of either neomycin sulphate (NS), povidone iodine (PVP-I), chlorhexidine digluconate (ChD) or silver sulfadiazine (SS) into polystyrene plates (used as moulds) and removing the bulk of the water by freeze drying. The effect of the released antimicrobials against MRSA (NCTC 11940) was determined *in vitro* using a modified Franz diffusion cell. The dissolution medium of the receptor chamber was a stirred phosphate buffer solution (pH = 7.4) inoculated with 5×10^5 cfu/ml of MRSA at 37 ± 0.5°C. Antimicrobial wafers were placed on top of a cellulose membrane (12–14 kDa) in contact with the inoculated medium. Samples (200 μl) of the dissolution medium were taken at 0-, 2-, 4-, 6-, 8- and 24-h intervals and each diluted to subinhibitory levels, 10-fold down to 10^{-5}, in 0.9% NaCl solution. Dilutions were plated on agar plates (5 μl × 20 volumes) and incubated at 37°C for 24 h. After incubation, bacterial colonies were counted and the original cell count (cfu/ml) was calculated. Results were compared using an established disc diffusion method.[1]

Results and Discussion

Compared with the placebo wafer (control), the MRSA level decreased dramatically over a 24-h period (Figure 1). PVP-I and ChD displayed a four-log reduction of MRSA within 5–6 h and 7–8 h, respectively, whereas NS took between 8 and 24 h. The insoluble nature of SS limited its passage through the membrane from wafer to dissolution medium. Regrowth observed with PVP-I was probably due to the instability of this antimicrobial.

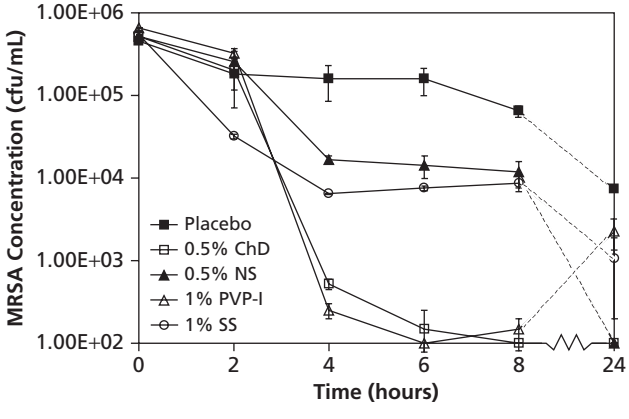

Figure 1 Concentration of methicillin-resistant Staphylococcus aureus bacteria in dissolution medium as a function of time for karaya wafers containing four different antimicrobial compounds ($N = 4$, SD).

Conclusion

The release of antimicrobials has a profound effect upon the viability of planktonic MRSA. Results confirmed that these wafers delivered the necessary antimicrobial effect. The modified Franz diffusion cell represented a superior in-vitro method, compared to disc diffusion, for evaluating the efficacy of antimicrobial wafers.

Reference

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28
Enhanced biotransformation of terpenoids in plant cell cultures using β-cyclodextrin

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Introduction and Objectives

The objective of this study is to use β-cyclodextrin (β-CD) to enhance biotransformation of terpenoids using plant cell cultures. The use of cyclodextrins (CDs) can overcome some important limitations in biotransformation of terpenoids, which include short incubation periods for exogenous substrates, toxicity of many monoterpenes to plant tissue, high turnover or degradative capability and volatility of terpenoids. CDs and their derivatives are capable to form inclusion complexes with terpenoids and, therefore, reduce toxicity, volatility and hydrolysis of precursors. They can also increase product yield through protection of formed products.

Method

Suspension cultures of *Peganum harmala* were initiated from callus and maintained in liquid Murashige and Skoog media[1] containing 3% w/v sucrose, 5 mg of ascorbic acid/l, 1.0 mg of 2,4-dichlorophenoxyacetic acid (2,4-D)/l and 0.1 mg of kinetin/l. A sample terpenoid, geranyl acetate (GOAc), was aseptically introduced into *P. harmala* normal suspensions and suspensions containing 1 and 2% w/v β-CD to give final concentrations of 100, 200 and 400 ppm. A time-course study of biotransformation was obtained by calculating the concentration of substrates and products in the cell cultures using gas chromatography (Finnigan Focus).

Results and Discussion

Plant cell cultures of *P. harmala* converted GOAc to geraniol (GOH). GOAc levels, in the absence of β-CD,

decreased rapidly from 100 and 200 ppm to 10.9 and 26.9 ppm, respectively, in the first 2 h, and GOH levels increased to 80 ppm in the first hour and decreased to 30 ppm after 2 h, which subsequently decreased to very low levels in the first 8 h. Feeding of GOAc to cultures containing β-CD increased levels of both substrate and product and extended the biotransformation time from 8 h in direct feeding to 72 h. GOAc remained at higher levels, 171, 105 and 44 ppm with 400, 200, 100 ppm, respectively, after 24-h incubation time. Also, high levels of GOH were obtained; the levels of GOH increased from 3 to 40 ppm and from 5 to 98 ppm with 100 and 200 ppm GOAc, respectively, compared with feeding without β-CD. The inclusion complex formed extended the biotransformation of GOAc to GOH and also protected the cells from toxicity of substrate, and the cells appeared healthy and viable, even with the highest concentration, 400 ppm, in contrast to feeding without β-CD, in which the cells suddenly died at high concentration of substrate.

Conclusions

β-CD greatly extended concentrations of both substrate and product over extended periods of time at somewhat constant levels especially with higher concentration, namely 2% β-CD. Also, β-CD enhanced biotransformation of terpenoids, by decreasing availability of substrate in the suspensions, through inclusion complex formation, thus reducing its toxicity on plant cells and allowing for increased amounts of substrate to be fed without any harmful effect to the cells.

Reference

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29
Amphiphilic graft copolymer of hyaluronic acid and polylactic acid for pulmonary delivery of Amphotericin B

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Introduction and Objectives

Delivery of antifungal agents to the site of infection has gained favour for treatment of pulmonary fungal infections, potentially enhancing therapeutic activity and reducing toxicity. The main objective of this study was to explore amphiphilic graft copolymers of hyaluronic acid (HLA) and polylactic acid (PLA) for solubilisation and lung delivery of hydrophobic amphotericin B (AmB).

Method

Graft copolymers of HYA-PLA were synthesised as reported earlier. Polymeric carriers containing AmB were prepared by dialysis. The AmB in dimethyl sulfoxide (DMSO) was added to HYA-PLA dissolved in a mixture of dimethyl formamide (DMF) and water. This solution was introduced into the dialysis tube and dialysed against deionised water for 24 h to remove organic solvents. Micelles were probe-sonicated and filtered through 0.45-μm filters to remove unentrapped AmB. Micelle surface charge was determined using a Malvern ZetaSizer (Malvern Instruments Ltd, Worcestershire, UK),

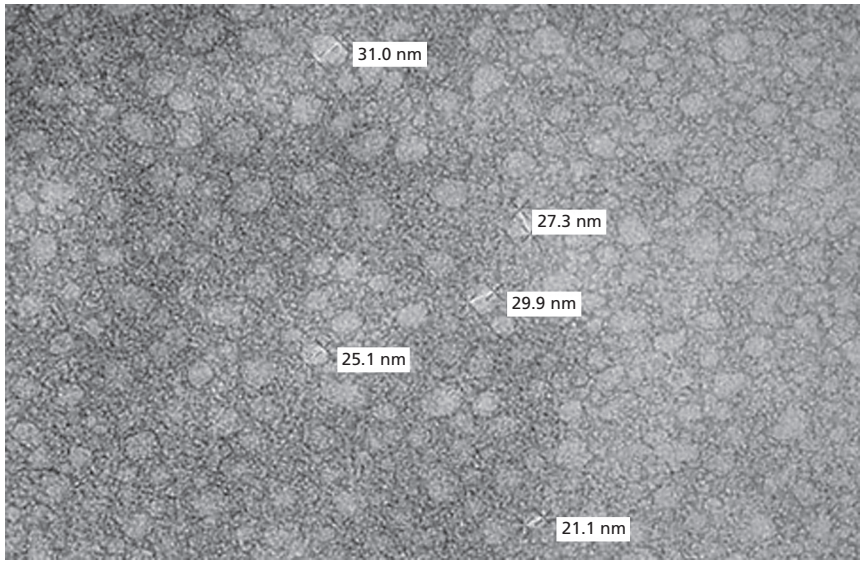

Figure 1 Transmission electron micrograph of hyaluronic acid and polylactic acid (HYA-PLA) micelles loaded with Amphotericin.

and micelle-associated AmB was determined using UV spectroscopy. The antifungal activities of formulations were assessed against *Candida albicans* by microdilution susceptibility testing in 96-well microtitre plates. Haemolysis method was used to establish the safety of these carriers for AmB delivery. Micelles (5 ml) were placed in a Pari LC Star (Pari GmbH, Starnberg, Germany) nebuliser attached to a TurboBoy N compressor (Pari GmbH) and nebulisation undertaken to 'dryness' into a twin impinger (TI). The AmB output and deposition in the fine particle fraction (FPF) (i.e. lower stage of the TI) were determined by UV analysis.

Results and Discussion

Results indicate that amphiphilic HYA-PLA can solubilise AmB during self assembly of the graft copolymer (Figure 1). Up to 50% of drug loading efficiency was obtained with 25% w/w theoretical AmB loading. The surface charge of drug-loaded polymeric carriers was negative. Polymeric carrier-loaded AmB exhibited antifungal activity against *C. albicans* comparable to Fungizone (AmB desoxycholate; Sigma Aldrich, UK). Haemolytic studies showed that encapsulation of AmB in HYA-PLA carriers reduced drug toxicity up to 20 μg/ml, whereas AmB in Fungizone was haemolytic at less than 10 μg/ml. Following nebulisation, the FPF of AmB was 58% AmB. Further in-vitro studies are ongoing with regard to stability and toxicity of these micellar systems.

Conclusion

These studies show that novel HYA-PLA polymer can be useful for solubilisation and delivery of hydrophobic drugs to the lung.

Short Papers in Drug Delivery

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Preparation of polymeric microspheres as an ophthalmic drug delivery system for brimonidine tartrate

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Introduction and Objectives

A dramatic increase in the incidence of eye diseases, such as age-related macular degeneration, and inadequate therapeutic control has led to the search for advanced methods of sustained and targeted drug delivery. The development of polymeric devices, matrix implants and microspheres has facilitated the prolonged controlled release of therapies to the target sites. The aim of this study is to prepare brimonidine tartrate–loaded microspheres, which possess suitable characteristics for development into an implantable device that will later exploit the binding properties of ocular melanin to influence the release properties.

Method

Microspheres were prepared using a solvent evaporation technique for encapsulation developed from those methods described in the literature.[1–3] Resomer RG502 (Boehringer Ingelheim, Ingelheim, Germany) (PLGA; ~50 : 50 D,L-lactide : glycolide) was dissolved in various nonhalogenated solvents after which a known quantity of brimonidine tartrate was added. In each case, this solution was added to the stirred aqueous continuous phase containing polyvinyl alcohol or Pluronic F-127. The emulsion was stirred for 3 h at room temperature after which the microspheres were collected by filtration or centrifugation and dried under vacuum. The collected microspheres were assessed for size and shape by optical light microscopy, and the size distribution was determined using a zeta-sizer. Percentage yield was calculated, and the encapsulation efficiency was determined by dissolving a known quantity of the microspheres in dichloromethane and extracting the brimonidine in 0.001 M tartaric acid. The aqueous layer of extraction was analysed for drug content using UV spectroscopy. Determination of release profiles was performed using an established method.

Results and Discussion

A range of microspheres were successfully prepared, which possessed different physical properties. An example of one of the batches of microspheres prepared from ethyl formate and a 0.5% w/w Pluronic F-147 aqueous phase is shown in Figure 1. A uniform size range was found where the average diameter of the particles was 7 μm, and each possessed a smooth spherical shape, although a more detailed investigation into their surface characteristics needs to be performed. This was true for all the microspheres prepared although problems with particle aggregation were encountered with the acetone solvent system due to their submicron size. It was found that as the average particle size of the microsphere decreased, the encapsulation efficiency decreased, especially in the case of those prepared from acetone where it was believed that their small size inhibited efficient encapsulation. Reduction in particle size also increased the rate of release. Further optimisation is ongoing to find the appropriate balance between particle size, encapsulation efficiency and release profile.

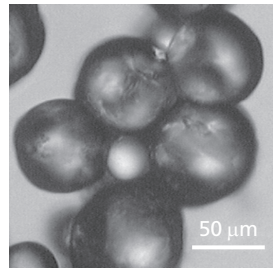

Figure 1 Optical light microscopy image of microspheres prepared from ethyl acetate.