Enhancing Transcutaneous Drug Delivery: Advanced Perspectives on Skin Models

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Skin acts as a dynamic interface with the environment. Pathological alterations in the skin barrier are associated with skin diseases. These conditions are characterized by specific impairments in epidermal barrier functions. Despite its protective nature, the skin can be a relevant route of drug administration, both for topical and transdermal therapy, allowing for improved drug delivery and reducing the incidence of adverse reactions. This manuscript reviews transcutaneous drug delivery as a strategy for treating localized and systemic conditions, highlighting the importance of skin models in the evaluation of drug efficacy and barrier function. It explores advances in in vitro, ex vivo, in vivo, and in silico models for studying cellular uptake, wound healing, oxidative stress, anti-inflammatory, and immune modulation activities. Disease-specific skin models are also discussed.

Keywords: Barrier function, Efficacy assessment, Skin diseases, Skin models, Skin physiology

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SKIN BARRIER PHYSIOLOGY AND PATHOLOGY: GENERAL CONSIDERATIONS

Skin physiology ensures the effective functioning of this interface organ as a robust physical barrier against the external environment. Besides its vital barrier function, the skin also plays a pivotal role in maintaining body temperature, processing sensory stimuli, interacting with the immune system, absorption, and excretion (Jiao et al, 2022). Among the different layers of the epidermis, the stratum corneum (SC)—the most superficial layer—distinguishes itself through its essential role in maintaining the skin's physical barrier function. This layer is composed of interconnected corneocytes and an extracellular bilayer—lipid matrix composed mainly of ceramides (40−50 wt%), free fatty acids (10−20 wt %), and sterols (25 wt%) (Jiao et al, 2022; Schmitt and Neubert, 2018). The organization of the SC is commonly described as a "bricks and mortar model", in which corneocytes (the bricks) are embedded in a specialized lipid matrix (mortar) (Elias, 1983). Each corneocyte is surrounded by a protein shell containing, among others, loricrin, involucrin, and FLG, which are covalently bound to lipids. The correlation between barrier dysfunction and the down-regulation of these molecules was recently reviewed (Furue, 2020).

Overall, the epidermis ensures a permeable, antimicrobial, antioxidant, immune response and photoprotective barrier (Almeida et al, 2022; Knox and O'Boyle, 2021; Sahle et al, 2015; Segre, 2006). The skin barrier function highly depends on the maturation process of keratinocytes and differentiation from the stratum basale to the SC as well as the composition and structure of the lipid matrix, particularly in terms of the quantitative and qualitative composition of ceramides (Almeida et al, 2022; Schmitt and Neubert, 2018). Structural features of the SC lipid matrix constrain water transport, by reducing transepidermal water loss and maintaining the water homeostasis in deeper layers of the skin. It also contributes to the reduction of the diffusion rate by approximately 50%, compared with phospholipid membranes (Schmitt and Neubert, 2018), thereby hampering the permeation of external xenobiotics. In addition, the permeation of drugs for topical or transdermal administration is regulated by the competent epidermal barrier. SC is thus recognized as the main mediator of permeability (Proksch

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Abbreviations: 2D, 2-dimensional; 3D, 3-dimensional; 4-HNE, 4-hydroxynonenal; AD, atopic dermatitis; BA, bioavailability; BCC, basal cell carcinoma; BE, bioequivalence; cSCC, cutaneous squamous cell carcinoma; ECM, extracellular matrix; H_2O_2, hydrogen peroxide; IHC, immunohistochemistry; IVPT, In vitro skin permeation test; NMSC, nonmelanoma skin cancer; RHE, reconstructed human epidermis; SC, stratum corneum; SCC, squamous cell carcinoma; TCI, topical calcineurin inhibitor

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et al, 2008), and its maintenance is essential for preserving its functionalities of defence and dehydration prevention (Feingold, 2012).

In a functional skin barrier, keratinocytes are connected to each other by intercellular junctions: adherens junctions that create a transcellular network (Tinkle et al, 2008); tight junctions that correspond to occluding junctions and are associated with the regulation of inside-out water fluxes (Bäsler et al, 2016); gap junctions that allow direct communication between the cytosols and are responsible for exchange of ions and metabolites (Meşe et al, 2007); and desmosomes, composed of the transmembrane proteins (cadherins, desmogleins, and desmocollins) to which keratin filaments bind. Desmosomes morphology changes in the SC, receiving the designation of corneodesmosomes (Johnson et al, 2014).

Altering the barrier can trigger various events that may culminate in the onset of skin diseases. Table 1 summarizes the main barrier impairments often observed in some exemplary skin conditions.

Melanocytes constitute the second most crucial cell type in the epidermis. These cells are primarily situated in the basal layer of the epidermis at the dermal–epidermal junction. Their main function involves the synthesis of melanin, encompassing both pheomelanin and eumelanin, which serves as a protective mechanism against UV-induced DNA damage, photoaging, and the development of skin cancer. Essential enzymes in melanogenesis include tyrosinase, TRP1, and TRP2, with tyrosinase acting as a limiting factor (Ito and Wakamatsu, 2008). The subsequent transfer of melanin to keratinocytes occurs through specialized organelles known as melanosomes, strategically distributed around the keratinocyte nuclei. Pigmentary disorders encompass a heterogeneous group of conditions manifesting with varying degrees of hyperpigmentation and/or hypopigmentation (Gillbro and Olsson, 2011). Although there is no direct evidence linking changes in melanocytes to alterations in the skin barrier, impaired melanocyte function can increase susceptibility to UVR damage, potentially leading to barrier impairment (Vocetkova et al, 2020).

REGIONAL AND TRANSDERMAL DELIVERY

The terms topical and transdermal deliveries are often erroneously used interchangeably. A topical medication is intended to have an effect at the site of application, not resulting in significant drug concentrations in the blood and other tissues, and causes fewer adverse reactions. Examples of topical medications include antibiotics for skin infections, corticosteroids for skin irritation, and some anesthetics. Thus, topical drug delivery is mostly used to target a localized area close to the application site, including deeper structures such as the hypodermis or joints, or for pathological skin disorders. Topical corticosteroids can be used to treat skin inflammation and some of the pruritic sensations in disorders such as dermatitis (Axon et al, 2021). In the context of rheumatic diseases, the topical administration of nonsteroidal anti-inflammatory drugs such as diclofenac or ibuprofen is used to alleviate osteoarthritis and rheumatoid arthritis by reducing pain and inflammation in the tissues beneath the application zone (Bariguian Revel et al, 2020).

Beyond the locally restricted effects, the skin also provides an administration pathway for systemically acting drugs. Transdermal delivery of medication is a widely used, successful method to treat several diseases (Dhillon, 2011). Transdermal medications are intended to have an effect in areas of the body away from the site of application, and the extent of skin permeation of a compound may depend on the permeant physicochemical characteristics and/or on the formulation (Schurad et al, 2022). Diffusional resistance imposes the first criterion for a successful transdermal candidate: transdermal drugs must be pharmacologically potent, requiring therapeutic blood concentrations in the ng/ml range or less (Prausnitz and Langer, 2008). The second criterion is that the SC is very selective with respect to the type of molecule. Not all molecules that are currently being formulated to cross the skin satisfy Lipinski's rule of 5: a molecular weight <500 Da, <5 hydrogen bond donors, <10 hydrogen bond acceptors, and a partition coefficient (log P, lipophilicity measurement) <5.1 (Choy and Prausnitz, 2011). This happens because several strategies have been developed to enhance through-the-skin delivery. Passive penetration enhancement can be achieved by manipulation of the formulation, by increasing the thermodynamic activity of the drug in formulations, by drug modification, or by using chemical penetration enhancers (Gupta et al, 2019). There is also a possibility to apply active methods, which involve the use of external energy to act as driving force and/or to reduce the barrier function of the SC (Ramadon et al, 2022). The extent of skin permeation of a compound may depend on the route of absorption. There are 3 pathways that can be involved in the transdermal permeation of chemicals: through the intercellular lipid domains in SC, through the skin appendages, and through the keratin bundles in SC (Schaefer et al, 2011). However, most of the commercialized transdermal delivery systems are based on passive diffusion through the intercellular route (Wiedersberg and Guy, 2014). In recent years, with the development of nanostructured carriers for skin delivery, the appendageal route of penetration, specially through hair follicles, has received an increased research interest (Patzelt and Lademann, 2020). However, only a few reports present the introduction of appendageal structures such as hair follicles within tissue-engineered skin models (Motter Catarino et al, 2023).

Transdermal administration can address safety and efficacy issues in disorders such as cardiopathies and endocrine disorders, which usually require oral systemic drugs (Nissinen, 1991). Angina pectoris is frequently treated with nitroglycerine ointments or patches (Thadani and Lipicky, 1994). As an alternative to oral drugs, transdermal hormone replacement patches or gels (eg, oestrogen or testosterone) are applied to administer systemically acting hormones (Kopper et al, 2009).

The location of the therapeutic target, the desired therapeutic impact, the drug's physicochemical qualities, and the overall treatment plan for a particular disease all influence the choice of delivery method (Hadgraft, 2004). Despite the existence of studies establishing correlations between in vitro, ex vivo, and in vivo data, there is a lack of studies with correlations to human data (Godin and Touitou, 2007).

Table 1. Skin Diseases Associated with Barrier Impairment

Disease	Characteristics	Barrier Impairment	References
Atopic dermatitis	Dry and scaly skin (nonlesional skin), which frequently develops into eczema (lesional skin). Characterized by hyperkeratosis, spongiosis (intercellular edema of the epidermis), and inflammation	Decreased level of ceramides	Bäsler et al (2016), Boguniewicz and Leung (2011), Bouwstra et al (2023), Dainichi et al (2014), and Sasaki et al (2024)
		TEWL: Nonlesional: slightly increased TEWL. Slightly increased uptake of polyethylene glycols of various molecular weights Lesional: more pronounced increase of TEWL and glycerol/SLS (1,3 distearoyl-2-linoleoyl-*sn*-glycerol) uptake Electrical impedance spectroscopy detects skin barrier dysfunction	
Psoriasis	Erythematous papules and plaques with a silver scale. Histologically characterized by parakeratosis, hyperkeratosis, absence of granular cell layer, elongated rete ridges, dilated blood vessels, and an infiltrate consisting of lymphocytes and polymorphonuclear leukocytes	Decreased level of ceramides	Choi et al (2021), Čuříková-Kindlová et al (2021), and Orsmond et al (2021)
		No change in FFA level	
		TEWL: Nonlesional: no alteration Lesional: increased TEWL and increased uptake of triamcinolone acetonide and minerals	
		Increased dermal vascularity and keratinocyte proliferation and proinflammatory cytokine secretion: TNFα, IL-6, IL-17, and IL-23	
Xerosis cutis	Scaly and rough skin surface, dry skin	Decreased level of ceramides	Fluhr et al (2024) and Murphy et al (2022a)
		Dehydration of the SC Reduction of skin elasticity and mechanical properties	
Ichthyosis (vulgaris)	Generalized, fine scaling that spares the flexures. Often associated with palmar hyperlinearity and atopic dermatitis	Decrease or absence of FLG	Vahlquist and Törmä (2020)
		Decreased level of acylceramides and FFA Absence of keratohyalin granula Hyperkeratosis Increased TEWL Increased hexyl-nicotinate penetration Absence of NMF	
		Association with atopic disorders and increased IgE	
Bullous pemphigoid	Subepidermal blisters	Keratinocyte death and sustained localized inflammation due to IgG autoantibodies directed to human BP180; BP230 resulting in cellular cytotoxicity and subepidermal blistering	De Benedetto et al (2012) and Stevens et al (2019)
Pemphigus vulgaris	Suprabasilar acantholysis	Acantholysis of keratinocytes, inflammation, skin barrier disruption, and further intraepidermal skin blistering due to IgG autoantibodies directed to human desmoglein (DSG1, DSG3), resulting in cellular cytotoxicity	De Benedetto et al (2012) and Stevens et al (2019)
Epidermolysis bullosa pruriginosa	Anchoring fibril abnormalities	Splitting at the dermal–epidermal junction and skin barrier disruption	De Benedetto et al (2012) and Stevens et al (2019)

Abbreviations: DSG, desmoglein; FFA, free fatty acids; NMF, natural moisturizing factor; SC, stratum corneum; SLS, sodium lauryl sulphate; TEWL, transepidermal water loss.

Moreover, data on the role of excipients, either enhancers or retarders on skin delivery of actives, in skin formulations are scarce (Patel et al, 2021).

SKIN MODELS: AN OVERVIEW OF IN VITRO, EX VIVO, IN VIVO, AND IN SILICO MODELS

In vitro models

Two-dimensional (2D) monolayer cultures, reconstructed human epidermis (RHE), and full-thickness models are examples of in vitro models. The latter mimics skin function and is therefore particularly helpful for drug administration. It also offers multiple cell types (including keratinocytes, fibroblasts, melanocytes, endothelium, or immune cells), 3-dimensional (3D) design (eg, full-thickness model, skin-on-chip model), and toxicity assessment in a more realistic setting. Although they still lack the native architecture of the skin, full-thickness models offer flexibility and the opportunity for long-term research.

The 2D monolayer culture models. Cells can be grown as a monolayer on solid flat surfaces, creating the simplest and most cost-effective cell-based skin model (Poumay and Faway, 2023). Initially, human keratinocyte monolayer cultures were established from primary cells obtained from the foreskin of newborns (Rheinwald and Green, 1977). These 2D culture models are often produced using commercially available cell lines (eg, HaCaT, NIH/3T3) and are employed for preliminary drug screening.

RHE. RHE models, also known as human epidermis equivalents, are composed of human keratinocytes arranged in stratified layers that closely resemble the epidermis's structure (Figure 1). They are frequently used to evaluate skin barrier function, permeability, drug absorption, irritation, and toxicity. EpiDerm and SkinEthic are examples of commercially available options (Netzlaff et al, 2005; Wang et al, 2023b).

Full-thickness models. These models (known as human skin equivalents) go beyond 2D monolayer cultures and RHE models by incorporating both epidermal and dermal components (Oh et al, 2013). They include a dermal-like layer, usually composed of human fibroblasts in a collagen matrix. Melanocytes added to the epidermis and endothelial cells incorporated into the dermis can be used to create pigmentation and vascularization in full-thickness skin models. These characteristics improve the model's realism, allowing the study of drug interactions within deeper skin layers, drug permeation, and absorption.

Skin-on-chip models. Their main strength resides in combining dynamic culture conditions and the capability to engineer the human skin microenvironment, allowing the manipulation of the extracellular matrix (ECM) architecture, fluid flow, and mechanical cues. Recent advances in skin-on-chip models include vascularization and immune system integration (Costa et al, 2023; Nitsche et al, 2022). Skin cell migration and proliferation studies have been conducted using microfluidic chips. In addition, studies have examined vascular smooth muscle cell migration as well as cell–cell and cell–ECM interactions. Research has also focused on endothelial cell migration and sprouting in response to GF gradients and shear stress (Costa et al, 2023; Dellambra, 2019). With the use of multilayered designs, perfusion, and enhanced cellular function, these sophisticated in vitro systems enable more precise drug delivery and toxicological research.

Ex vivo models

Ex vivo skin models involve explants or biopsies obtained from animals or human reductive surgeries. Skin biopsies maintain the in vivo architecture and cellular interactions of human skin. These models have been employed to investigate chemical toxicity (Nakamura et al, 1990), inflammation, wound healing (Guilloteau et al, 2010; Xu et al, 2012), infections (Corzo-León et al, 2019), and barrier repair (Danso et al, 2015). Skin explants can be treated with compounds, and various endpoints/parameters, such as cytokine production, can be measured (Shannon et al, 2022; Zhou et al, 2023). Different methodologies have been applied to skin explants for the identification of immune cells and proteins, for the measurement of lipid composition, or for the assessment of gene expression.

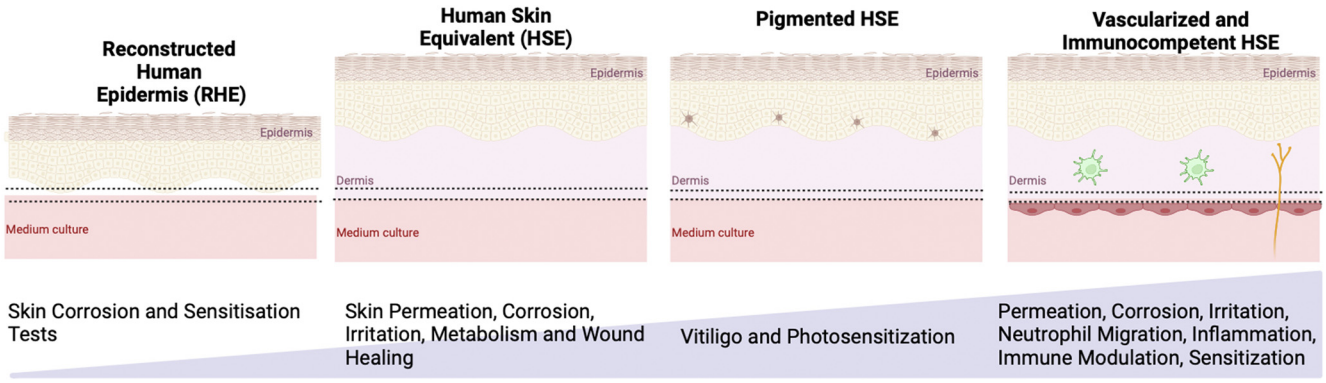

Figure 1. Schematic representation of the 3D in vitro skin models, with respective research applicability. From left to right, increased biological complexity is obtained. These skin tissue models can be cultured with different levels of biological complexity, RHE being the simplest one, consisting only of keratinocytes and with limited applicability. HSE (corresponding to full-thickness bilayered reconstructed skin model) contains fibroblasts integrated into a collagen I scaffold to create a dermal compartment. HSE models complemented with melanocytes and with immune cells have the potential to revolutionize drug delivery and toxicological research. 3D, 3-dimensional; HSE, human skin equivalent; RHE, reconstructed human epidermis.

Pig skin has been used as a human skin surrogate for ex vivo assays because it provides a quantitative and reproducible model for topical drug bioavailability (BA) studies, with results comparable with those observed in human subjects (Herkenne et al, 2006). Nevertheless, recently adopted guideline on quality and equivalence of locally applied, locally acting cutaneous products (EMA, 2018; coming into effect on April 2, 2025) states that the use of ex vivo animal skin is not yet sufficiently established to provide pivotal evidence and requires the use of ex vivo adult human skin for therapeutic equivalence studies.

Human skin explants can be cultured to serve as ex vivo models for topical therapeutics investigation. Neil et al (2020) reported the successful use of cultured human skin for up to 9 days without any adverse physiological consequence, allowing for the mounting of tissue explants into Franz cells for the topical application of formulations.

In vivo animal models

Mouse models of skin inflammation. Transgenic mice, BALB/c mice, humanized mice, SCID mice, 129/Sv mice, or mice with induced skin inflammation provide in vivo platforms to study immunologic, cellular, or molecular pathways. These models allow researchers to assess the effects of substances or drugs on inflammatory mediators, immune cell infiltration, and overall skin health through intradermal injection, subcutaneous injections, or topical application (Miyagawa et al, 2010; Pivetta et al, 2018). Although human skin is much thicker and more complex than mouse skin and differs in epidermal immune composition, there is a recognized applicability of mouse models to study skin delivery in inflamed skin (Medetgul-Ernar and Davis, 2022).

Zebrafish embryo models. Zebrafish embryos, owing to their transparency, offer a unique opportunity to visualize and study inflammatory responses in vivo or skin pigmentation (Mutalik et al, 2024). They are particularly useful for high-throughput screening of substances for their effects on skin inflammation (Qu et al, 2023).

In silico models

In silico models such as molecular docking and simulations have been employed to estimate the effect of therapeutic approaches, predicting the interactions between compounds and specific molecular targets (Eyerich et al, 2019). Although not a substitute for experimental models, they can guide researchers in designing experiments and prioritizing substances for further testing. These models collectively contribute to a comprehensive understanding of how compounds affect skin properties. Different types of in silico models can be developed but are dependent on the availability of high-quantity data. In silico models use data from in vitro human skin studies from different databases (European Food Safety Authority, 2017). Even counting with considerable information about drug distribution within the skin after infinite dosing, lack of data for finite dosing exists as well as for semisolid drug preparations (Selzer et al, 2013).

Researchers often combine different models to validate findings across various experimental systems, increasing the robustness and relevance of their conclusions. The model of choice depends on the specific research objectives, the nature of the substance being tested, and the desired level of complexity in the experimental setup.

Figure 2 summarizes the approaches available to mimic skin physiology, for topical and transcutaneous drug application research. Advances toward the development of in silico, in vitro, and ex vivo skin models have been proposed in the last years, from the more simplistic 2D cell monolayers to more complex and biologically relevant 3D systems. However, not all accurately represent normal human physiology. In parallel with high-throughput models with limited physiological relevance, the generation of skin-on-chip models, consisting of in vitro 3D vascularized skin models with dynamic perfusion and microfluidic devices, enables a more physiological transport of nutrients.

SKIN DELIVERY ASSESSMENT

Transcutaneous penetration assessment involves the evaluation of parameters and measurement of compounds through the skin. This process serves multiple purposes, including monitoring skin health, evaluating drug delivery, and investigating physiological changes. This route of application allows for the systemic delivery without the need for injection or ingestion, providing a noninvasive alternative for drug administration. However, one of the most critical determinants of the efficacy of topical and transdermal formulations is the permeation of drugs across the epidermal barrier and subsequent penetration into the deeper layers of the skin. Tape stripping is commonly used to establish in vitro and ex vivo the drug concentration profile across the upper epidermis (Moser, 2001). Adhesive strips (eg, D-Squame Stripping Discs) are used to sequentially sample the SC, permitting the calculation of diffusivity and solubility of the permeant within this layer by determining the drug amount and thickness of SC removed in each tape strip (Ilić et al, 2021; Pellett et al, 1997; Lademann et al, 2009). Similar outputs can be obtained by performing skin surface biopsies with cyanoacrylate, which can provide additional data

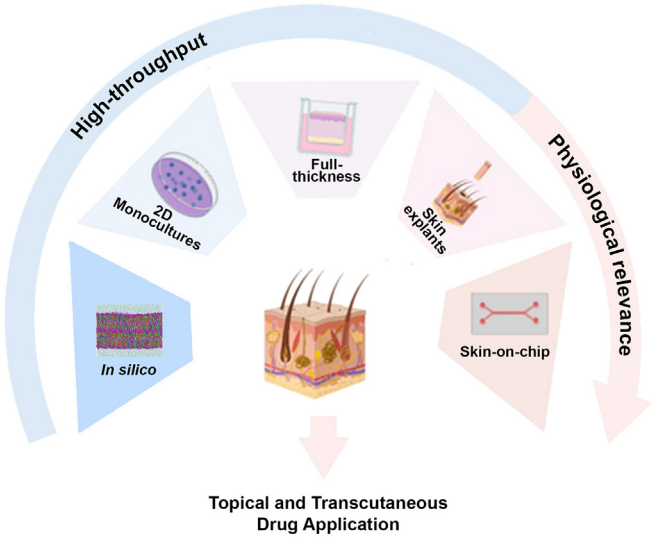

Figure 2. Schematic representation of the approaches to mimic skin physiology for topical and transcutaneous drug application. This figure was created with BioRender (https://www.biorender.com/). 2D, 2-dimensional.

regarding drug content in the hair follicles (Abd et al, 2018; Zhai and Maibach, 1996). The high-performance liquid chromatography–thiobarbituric acid reactive substances–ex vivo SC method was proposed to evaluate oxidative stress in tape-stripped SC after exposure to UVR. This ex vivo analytical protocol combines liquid chromatography with the thiobarbituric acid reactive substances assay to assess the lipoperoxidation level in the SC (Marques et al, 2023).

Comparative clinical efficacy studies for skin-applied drugs are frequently hindered with proper quantification of expectedly low systemic drug levels, prompting the regulatory agencies to turn to the state-of-the-art research and gradually implement changes in their guidelines for demonstrating BA and bioequivalence (BE) (Miranda et al, 2023). Clinical efficacy studies also include other parameters, namely therapeutic effectiveness and comparative efficacy with that of other applied drugs toward similar goals, the dose–response, safety and patient-reported information.

Development of transdermal delivery systems continues to be an emergent area of research and innovation in pharmaceutical fields, aiming to improve drug delivery efficiency to a broad range of drugs that can be administered through the skin. However, these innovative (trans)dermal formulations, frequently associated with nanosystems, are attributed to complex microstructures that can add unexpected variability in their assessment (Isailović et al, 2016; Simões et al, 2018).

Beside clinical studies, current in vivo options for BA/BE assessment encompass the vasoconstrictor assay (exclusive to corticosteroid products) and several dermatopharmacokinetic methods, for example, tape stripping (Tabosa et al, 2022), or more invasive dermal open-flow microperfusion (Birngruber et al, 2022) or microdialysis (Zhang et al, 2017). In addition, spectroscopic methods, both in vivo and in vitro, raise high expectations, similar to confocal Raman spectroscopy (Zarmpi et al, 2023). Methods using radioactivity such as autoradiography can also be used, offering information on drug localization and penetration pathways (Grégoire et al, 2020).

In vitro skin permeation test (IVPT) using ex vivo human skin is considered an acceptable assessment method (Ilić et al, 2021). However, the study protocol is quite stringent, requiring full specification of the number of donors, inclusion/exclusion criteria for the excised skin's anatomical region, conditions of storage, justification of the applied skin preparation technique and the obtained skin thickness, and demonstration of skin integrity before and after the test (EMA, 2024). Nevertheless, even if all the conditions are met, researchers may face significant IVPT results variability. Therefore, it becomes apparent that the timely development and market placement of topical and transdermal products require additional surrogates for both human volunteers and their excised skin, possibly found in the realm of skin models.

All the mentioned assessment approaches (Figure 2) rely on either healthy adult subjects or human skin samples with intact integrity. Nonetheless, both the selected volunteers and the characteristics of the collected skin samples could significantly contribute to the variability of the results. Hence, skin models may prove to be a suitable platform for the noninvasive evaluation of a drug formulation's efficacy profile. The inclusion of specific disease skin models for the investigated drug would provide a comprehensive therapeutic profile of the (trans)dermal preparation (Figure 3).

EFFICACY ASSESSMENT ENDPOINTS

To probe the efficacy of different therapeutic agents, various endpoints may be considered.

Cellular uptake

Cellular uptake is crucial for drugs to reach their intracellular target, thereby underlying the efficacy of various cutaneous formulations. The main routes of cell internalization are the diffusion through the plasma membrane and endocytosis. Cellular uptake methods are mainly based on 2D and 3D cell cultures, which are exposed to fluorescent materials (drugs or labeled compounds) and internalization detected by fluorescence microscopy or flow cytometry. Moreover, various chemical endocytosis inhibitors (eg, chlorpromazine, hypertonic sucrose, 7-keto-cholesterol) may be added to cell

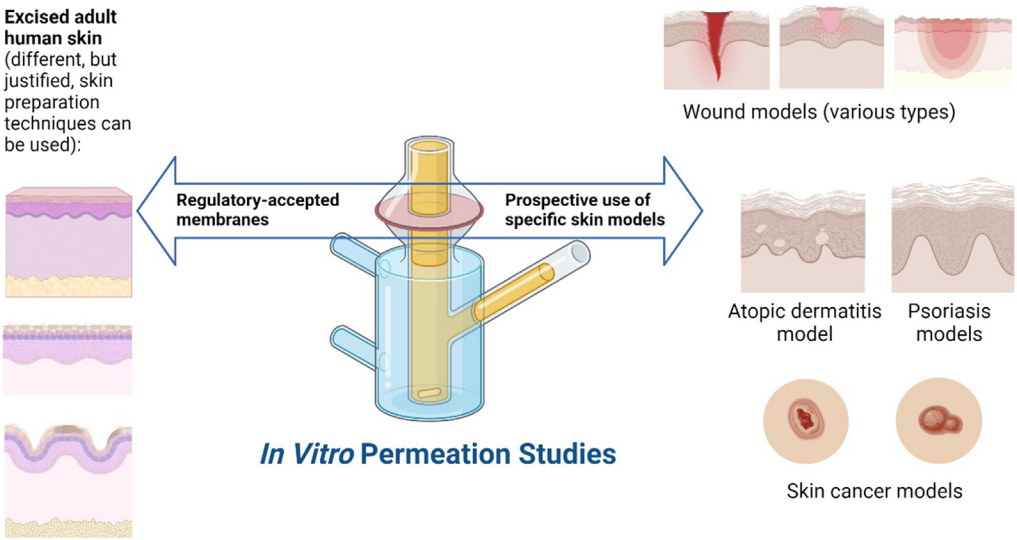

Figure 3. Current regulatory status versus research prospects of the IVPT. If combined with specific skin models, the IVPT method may provide more comprehensive data on efficacy and/or safety aspects of product application to diseased skin. Both pharmaceutical and cosmetic industry may benefit from such testing setups because cosmetic products may be applied by patients as well as healthy consumers. This figure was created with BioRender (https://www.biorender.com/). IVPT, in vitro skin permeation test.

cultures to dissect the uptake mechanisms. Recent data indicate that many of these inhibitors are nonspecific and display a cross-reactive nature; thus, the experiments must be well-designed to evaluate specific pathways (Li and Monteiro-Riviere, 2016; Rennick et al, 2021; Silva et al, 2017).

Intracellular ROS levels

ROS are chemically reactive molecules containing oxygen that include free radicals, such as superoxide anion ($O_2\bullet-$) and hydroxyl radical (•OH), as well as nonradical molecules such as hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$). To maintain homeostasis, antioxidants regulate the levels of the free radicals. However, an imbalance in the cell oxidative/antioxidative status generates excessive free radicals leading to oxidative stress. This results in damage to nucleic acids, proteins, and lipids, ultimately causing cell death, and chromosomal aberrations that directly or indirectly contribute to pathogenesis. Skin is exposed to a wide range of environmental ROS-inducing factors that can exacerbate skin aging, cause inflammation, or promote carcinogenesis. Cellular ROS can be measured directly using the nonfluorescent membrane-permeable 2′,7′-dichlorodihydrofluorescein diacetate, which is hydrolysed by intracellular esterase to 2′,7′-dichlorodihydrofluorescein and oxidized by H_2O_2 to the fluorescent 2′,7′-dichlorofluorescein that can be detected in 2D/3D skin models through fluorescence microscopy (Jordão et al, 2023; Pecorelli et al, 2021). H_2O_2 can be quantified in cell culture medium using 10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red, Ampliflu Red), which produces the red-fluorescent oxidation product, resorufin (Pecorelli et al, 2021). A similar system (ROS-Glo) in monolayers uses a derivatized luciferin substrate that produces bioluminescence (Khachatoorian et al, 2021). Superoxides can be detected using dihydroethidium, which is oxidized to the fluorescent ethidium bromide (Xu et al, 2020). CELLROX cell-permeant dyes exhibit fluorescence upon oxidation by intracellular ROS and subsequent binding to DNA, with detection through microscopy (Wang et al, 2023a). In addition to direct measurements, assessment of oxidative damage in experimental skin models is also a valuable tool. During lipid peroxidation, ROS oxidize membrane lipids, generating lipid hydroperoxides and aldehydes, such as malondialdehyde, 4-hydroxy-nonenal (4-HNE) and acrolein, all of which can be measured by immunofluorescence (Lecas et al, 2016; Verdin et al, 2019; Zucchi et al, 2022) or 4-HNE in protein lysates by ELISA (Pecorelli et al, 2021). Aldehydes can form modified proteins quantified by immunoblot (Zucchi et al, 2022) or using a protein carbonyl content assay, where their reaction with 2,4-dinitrophenylhydrazine results in the production and detection of 2,4-dinitrophenylhydrazine hydrazones.

Assessment of DNA damage can also be employed to quantify oxidative damage in skin models. The 8-hydroxy-2′-deoxyguanosine, the most common product of oxidative-mediated DNA modifications, is generated by hydroxylation of deoxyguanosine residues, detectable by immunohistochemistry (IHC) (Hakozaki et al, 2008) or ELISA (Kala et al, 2023). Similarly, thymidine glycol, another oxidative stress–induced DNA lesion, can be detected by IHC. Another common method is the detection of DNA double-strand break formation though phosphorylation of the histone protein H2AX to form γ-H2AX foci (Redon et al, 2009) that can be measured in several assay formats. Alternatively, increased levels of enzymes that repair ROS-induced endogenous DNA damage, such as 8-oxoguanine-DNA-glycosylase and apurinic/apyrimidinic endonuclease, can be measured.

Cells contain a large number of antioxidants to prevent or repair the damage caused by ROS as well as to regulate reduction–oxidative–sensitive signaling pathways. Assessment of either the enzymatic (eg, superoxide dismutase) (Xian et al, 2019) or nonenzymatic (eg, glutathione) antioxidant status can thereby be correlated to the extent of oxidative stress in skin models (reviewed in Katerji et al [2019]).

Anti-inflammatory and immune modulation activities

The anti-inflammatory activity and immune modulation activity of substances can be assessed by several in vitro and in vivo skin models that aim to simulate the complex interactions that occur in human skin. The most advanced skin-on-chip models were recently employed to assess the drugs' impact on inflammation and edema. The model comprised 3 distinct cell layers: HaCaT in the epidermis, fibroblasts in the dermis, and human umbilical vein endothelial cells forming the endothelium. Administration of dexamethasone, an anti-inflammatory steroid, resulted in a reduction of TNF-α–induced proinflammatory cytokines (IL-β, IL-6, and IL-8) and a decrease in edema, as demonstrated by permeability measurements and tight junction staining (Wufuer et al, 2016). In another study, a skin-on-a-chip device was created to mimic wound inflammation. Dexamethasone administration decreased the expression of IL-1β, IL-6, and IL-8 and increased endothelial cell binding. This device is a useful tool for preclinical screening and has the potential to identify potent anti-inflammatory therapies (Biglari et al, 2019).

Table 2 provides an overview of the various aspects considered when assessing the anti-inflammatory and immune modulation activity of substances on the skin, along with the corresponding parameters measured and common methodologies used for analysis.

The selection of specific assays and endpoints depends on the research goals, the nature of the substances being tested, and the specific skin conditions under investigation. A combination of in vitro, ex vivo, and in vivo approaches is often employed to obtain a comprehensive understanding of the anti-inflammatory and immune modulation activities of substances on the skin.

SKIN MODELS OF DISEASE

Assessment of drug or formulation effects on diseased skin is essential in drug development because it more accurately reflects the morphopathological modifications of the skin.

Atopic dermatitis

Topical therapies, such as glucocorticosteroids or topical calcineurin inhibitors (TCIs), have been the first-line treatment option for the management of mild-to-moderate atopic dermatitis (AD). mAbs blocking the IL-4/IL-13 receptor or Jak

Table 2. Assessment of the Anti-Inflammatory and Immune Modulation Activity of Substances on the Skin: Parameters and Methodologies

Activity	Parameters Measured	Methodology/Analysis	References
Cytokine secretion	Proinflammatory cytokines (IL-1, IL-6, TNF-α)	ELISA, PCR, immunohistochemistry	Angst et al (2008), Murphy et al (2022b), Pérez-Salas et al (2023), and Xiao et al (2020)
	Anti-inflammatory cytokines (IL-10)	ELISA, PCR	Lin et al (2021) and Pérez-Salas et al (2023)
Chemokine production	Chemokines	ELISA, PCR	Pérez-Salas et al (2023) and Sun et al (2021)
Cellular infiltration	Immune cells (macrophages, T cells)	Flow cytometry, immunohistochemistry	de Boer et al (2007) and Murphy et al (2022b)
Histological analysis	Microscopic examination	H&E staining, immunohistochemistry	Hügel (2002)
Barrier function	TEWL	TEWL measurement	Klotz et al (2022) and Uehara et al (2023)
	Skin hydration levels	Hydration measurement devices	Chen et al (2023) and Gidado et al (2022)
	Skin resistance to an electrical current reflects the barrier function	Electrical impedance spectroscopy	Baidillah et al (2024)
Oxidative stress	ROS	Fluorescent probes, enzyme assays	Nakai and Tsuruta (2021)
	Antioxidant enzyme activity	Enzyme assays	Shariev et al (2021)
NF-κB and MAPK signaling	Activation of NF-κB and MAPK pathways	Western blot, immunohistochemistry	Wang et al (2019)
Gene expression analysis	Inflammatory and immune-related genes	qPCR, microarray analysis, QuantiGene	Tang and Zhou (2020)
Inflammatory skin conditions	Disease-specific pathway modulation	Models (eg, psoriasis, atopic dermatitis)	Sarama et al (2022)
In vivo models	Systemic and local responses	Animal models, human studies	Beck and Leung (2000)
Clinical assessments	Erythema index, edema, subjective symptoms	Instrumental (eg, Chromameter, Mexameter, full-field laser perfusion imaging, etc) and/or visual assessment, patient questionnaires, clinical scores	Frew et al (2021), Jemec and Johansen (1995), and Qian et al (2015)

Abbreviation: TEWL, transepidermal water loss.

inhibitors are used for treatment of moderate-to-severe disease (Agache et al, 2021). Beside systemic treatments with biologicals (mAbs) or small molecules (Jak inhibitors), local application through topical or transdermal drug delivery approaches has been expanded. In addition, there is an increased focus on the restoration of a healthy skin microbiome using skin microbiome transplantations, prebiotics, or probiotics (Callewaert et al, 2021). Some of the treatments received approval from the United States Food and Drug Administration and European Medicines Agency, whereas other promising formulations are on the horizon. Ruxolitinib cream is a Jak1/Jak2 inhibitor, which can be applied topically for the treatment of mild-to-moderate AD. Ruxolitinib has been approved for patients aged ≥12 years. Additional novel Jak inhibitor formulations (delgocitinib ointment or cream) for the treatment of AD or hand eczema (phase IIb) are still under investigation (Worm et al, 2022). Crisaborole ointment, difamilast ointment, and roflumilast cream are relatively new topical treatments for mild-to-moderate AD. These therapeutic agents offer an alternative to glucocorticosteroids by inhibiting phosphodiesterase (mainly PDE4B) in patients from age 3 months (United States) or 2 years (European Union), avoiding some of the adverse effects of glucocorticosteroids.

The so-called Leiden epidermal model, in which the medium is supplemented with IL-4, IL-13, IL-31, and TNF-α, can be used to induce AD-like features in a full-thickness skin model (Danso et al, 2014). Full-thickness skin models of both AD and psoriasis were developed to assess the impact on keratinocyte immune function and barrier formation of tofacitinib pretreatment (Clarysse et al, 2019). To induce AD, 3D skin equivalents were stimulated with recombinant human IL-4 and IL-13, whereas to create psoriasis conditions, IL-17A, IL-22, and TNFα were used. Psoriasis and AD skin models can be thus induced by cytokine stimulation or by integrated T helper 1 CD4+ T cells, for both histology and function (Scheurer et al, 2024), thus reflecting several of the clinical features of these diseases.

Psoriasis

For treatment of mild-to-moderate disease, topical treatment with TCIs, vitamin D analogs, and tazarotene is standard of care. UVB phototherapy is used as well as systemic treatments with methotrexate; cyclosporin; mAbs targeting IL-17, IL-12, and IL-23; anti-TNFα; or Jak inhibitors (Drakos et al, 2024). Novel topical treatments contain active ingredients that specifically inhibit or modulate disease-related immune pathways, such as Jak inhibition, phosphodiesterase-4 inhibition, and aryl hydrocarbon modulation (Bissonnette et al, 2023). Tapinarof is a novel aryl hydrocarbon receptor–modulating agent. Tapinarof has been approved for the treatment of psoriasis and was shown to be beneficial for the treatment of moderate-to-severe AD in phase III trials from the age of 2 years (Paller et al, 2023). These treatments

block the release of disease-related cytokines, thereby preventing the ongoing inflammation (Ju et al, 2022; Takahashi et al, 2024).

As mentioned earlier, AD and psoriasis models have been developed in parallel in full-thickness skin models (Clarysse et al, 2019; Scheurer et al, 2024). Another model to study the interaction between keratinocytes and T cells was developed in a 3D microenvironment, on the basis of human skin equivalents with disease-relevant immune cells (CD4+ T cells, T helper 1 cells, or T helper 17 cells) (van den Bogaard et al, 2014). This strategy to populate 3D skin constructs with T cells enabled quantification of T-cell responses (Shin et al, 2020).Acne

Acne management has evolved through the search of novel treatments (clascosterone, trifarotene, and sarecycline) or through the development of better formulations of existing compounds (minocycline, tretinoin, tazarotene, benzoyl peroxide) (Auffret et al, 2022).

Several in vitro, ex vivo, or in vivo models have been established to evaluate drugs and formulations efficacy (Kanwar et al, 2018). In vitro 3D models mimicking acne-like skin are still scarce (Laclaverie et al, 2021). These combine sebum composition modification and invasion of *Cutibacterium acnes* and *Staphylococcus epidermidis* (Forraz et al, 2023), with the normal human keratinocytes used to generate the RHE.

Pigmentary diseases

Various hypopigmentation disorders, such as vitiligo, pityriasis versicolor, piebaldism, and oculocutaneous albinism, pose significant challenges. Vitiligo is an autoimmune-driven chronic depigmenting disease in which oxidative stress plays a pivotal role. Psoralen plus UVA phototherapy utilizing plant secondary metabolites, particularly furocoumarins, is a therapeutic approach (Gillbro and Olsson, 2011). However, it is crucial to acknowledge the phototoxic, photocarcinogenic, and photomutagenic nature of furocoumarins. Immunomodulators, such as cyclosporine and levamisole, represent alternative treatments for vitiligo. Hyperpigmentation disorders, including "Café-au-lait" macules, and post-inflammatory hyperpigmentation necessitate diverse treatment modalities. Hydroquinone, mequinol, retinoids, azelaic acid, and ascorbic acid are among the therapeutic agents employed, and many others have been tested (Shankar et al, 2014).

The de-epidermized dermis model, based on a native ECM and a basal membrane that facilitates melanocyte adhesion, has been used for a long time to demonstrate the effect of UVR and other stress factors on skin pigmentation (Cario-André et al, 2007). Different commercial pigmented skin models are available, with cells seeded on polycarbonate filter (SkinEthic, StratiCell) or on collagen gels (MelanoDerm, Melanoma skin model) (Gendreau et al, 2013). They have been used for testing the toxicity of cosmetics and for efficacy assessment in pharmaceuticals.

Wound healing

Various types of skin models have been used to assess the impact of drugs on wound healing. Among 2D cell culture models, the in vitro scratch test is usually used, aiming at evaluating the collective cell migration after creating a cell-free gap using a mechanical, thermal, or chemical stimulus. Overall, this assay provides information on the rate of gap closure when cells are exposed to different conditions, thereby being an important tool to evaluate the efficacy of wound-healing formulations (Jonkman et al, 2014; Martinotti and Ranzato, 2020; Saraiva et al, 2022). However, other assays, such as the barrier migration assay (Das et al, 2015, 2016) and the Boyden chamber assay (Chen, 2005; Monsuur et al, 2016), have also been successfully implemented. The 3D skin models, such as spheroids (PromoCell, 2022) and full-thickness models to mimic incisional, excisional, and burning wounds (Ansell et al, 2014; Schneider et al, 2021) also seem to be promising to evaluating the efficacy of wound-healing formulations. Animal models have been also explored to assess both acute and chronic wound healing as detailed elsewhere (Flynn et al, 2023; Saeed and Martins-Green, 2023). Furthermore, advances in in silico modeling to assess wound healing were recently reviewed (Abu Bakar et al, 2024).

Skin cancer

Skin cancer is the most frequent type of malignancy, with the ratio of occurrence of skin cancer to other cancer types reported at 1:3 (AlSalman et al, 2018), and its incidence is increasing (Guy et al, 2015). Nonmelanoma skin cancers (NMSCs), derived from keratinocytes, account for 95% of the malignant skin tumors (Duarte et al, 2018; Fijałkowska et al, 2021; Rezende et al, 2019), whereas melanoma, derived from melanocytes, is responsible for 1% (Bartoš and Kullová, 2017; Garbe et al, 2022; Hogue and Harvey, 2019).

Commercial human melanoma models, such as full-thickness melanoma skin constructs (MLNM-FT-A375 kit), containing A375 melanoma cells, or MLNM-FT-EXP (MatTek Life Sciences), containing the melanoma cells isolated from patients or other cell types, are currently available and can be used for cancer progression, melanoma genesis studies, or drug testing (Tanese et al, 2012). Different 3D melanoma models were reported, such as multicellular tumor spheroids (Baciu et al, 2022) and organoids (Lin et al, 2022; Pizzurro et al, 2021) or human skin planar reconstructs using bioprinted ECM and normal skin cells, coseeded with melanoma cells (Weng et al, 2021). Model complexity was increased by adding vasculature and immune cells to study melanoma progression, metastasis, or efficacy of different treatments (Nomdedeu-Sancho et al, 2023), including immunotherapies (Soares et al, 2020).

The most frequent NMSC is the basal cell carcinoma (BCC), accounting for more than 80%, followed by cutaneous squamous cell carcinoma (cSCC). BCC is characterized by a slow evolution, but it can lead to local tissue invasion and destruction, particularly the scleroderma subtypes (Roy et al, 2020), leading to frequent relapses, and can severely alter the QOL of the patients. cSCC is an aggressive cancer, whose evolution becomes invasive and metastasizes (Kumah and Bibee, 2023). Therapy of NMSC usually involves surgery, but other local therapies such as cryotherapy, topical 5-fluorouracil, and toll receptor inhibitor—imiquimod—can be used in early clinical stages, respectively radiotherapy, chemotherapy, and immunotherapy for advanced cases. A viable full-thickness model of BCC for experimental studies,

particularly the therapeutical drug testing, was obtained from cells derived from patients with BCC using a stromal self-assembly method (Roy et al, 2020). cSCC in vitro models can involve patient-derived cell models, especially for genomic studies and cancer progression, such as metastasis and lymph node involvement (Hassan et al, 2019; Kodack et al, 2017; Krause et al, 1981; Kumah and Bibee, 2023; Popp et al, 2000). Other models involve commercial cell lines derived from cutaneous and metastatic squamous cell carcinoma (SCC), used in genomic studies (Zhao et al, 2011), drug testing (Hail and Lotan, 2001), carcinogenesis (Farshchian et al, 2011), and tumor markers (Junttila et al, 2007; Moilanen et al, 2017). Tumor 3D models, such as spheroids obtained from A431 cells (Adhikary et al, 2013), were used for drug screening (Nunes et al, 2019). A recent study reported the use of 3D bioprinted tumors, such as unicellular spheroids of A431 cells, or tricellular, containing A431 cells, normal dermal human fibroblasts, and HaCaT cells, used for cetuximab testing (Kurzyk et al, 2024) and respectively nanomaterials toxicity, using multicellular tumor spheroids that contained SCC-25 and UPCI: SCC-154 cells (Santi et al, 2020). A novel 3D bioprinted skin tissue, containing A431 spheroids to mimic advanced/metastatic SCC, was engineered to test chemotherapeutics effectiveness (Browning et al, 2020).

CONCLUSIONS

The skin is an important route of drug administration and presents complex barrier properties as well as a risk factor for allergic sensitization, which makes it an organ under intense investigation. The search for adequate skin models has led to several skin surrogates and increasingly sophisticated structures to investigate skin diseases and new therapeutic targets. Increasing interest in alternatives to laboratory animals and stringent ethical regulations accelerated the development of skin models that mimic a disease situation. Tissue engineering techniques, bioprinting, and microfluidics have been implemented to find simple and affordable in vitro, ex vivo, and in silico assays. Topical drug delivery approaches have benefited from models developed to mimic the cellular and molecular events in specific skin diseases. The challenge is to improve high-throughput characteristics while maintaining reliability. In the case of permeation assays, the methodologies are somewhat standardized and regulated. However, in the case of studies evaluating therapeutic efficacy, for the same skin disease, there are several possible models, and a degree of harmonization is still required. Complementarity between basic research, modeling the complexity of human skin, and clinical applications would be of great value for a better preclinical testing of novel transcutaneous therapeutics.

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CONFLICT OF INTEREST

The authors state no conflict of interest.

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DECLARATION OF GENERATIVE ARTIFICIAL INTELLIGENCE (AI) OR LARGE LANGUAGE MODELS (LLMs)

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