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Review

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Role of skin enzymes in metabolism of topical drugs

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Abstract

Topical drugs have gained a lot of interest with their massive market growth and are available in various dosage forms. Prodrug compounds of transdermal delivery systems can be very different and designed to convert into the form of active pharmaceutical ingredients (APIs) through enzymatic action once they enter the body. The skin, as an interfacial barrier between the body and surroundings, has demonstrated critical roles in metabolizing, filtering, and detoxifying to minimize certain side effects and improve the medication benefits of topically administered products. It is well recognized that the drug pharmacokinetics can be altered by the presence of skin enzymes driven by biotransformation reactions. To evaluate the effectiveness of a topical generic drug product, its safety, and bioequivalence with the reference one, models assessing enzyme metabolic activity are highly required for testing the amount of drugs that are metabolized or can potentially be metabolized in both healthy and compromised skin. Thus, knowledge of skin composition and enzyme expression levels is of paramount importance in mapping the relevant metabolism that may have occurred. Regulatory authorities have also been making efforts to develop efficient and harmonizable protocols to evaluate the metabolism of transdermal products. This review is a compilation of reported skin metabolizing enzymes, including their role in both drug metabolism and homeostasis regulation, along with their localization and quantification in skin equivalents (and/or membrane layers). Various aspects that potentially affect the skin enzyme metabolism study were also discussed with respect to drug development considerations.

Keywords: Topical drugs, skin enzymes, metabolism, drug-metabolizing enzymes, homeostasis, skin models, vitamins, drug development

INTRODUCTION

Global topical drug delivery has gained extensive interest in the pharmaceutical and dermatological industry, signifying a market growth from USD $109.1 billion in 2023 to USD $176.8 billion by 2030[1,2]. Topical drugs are medication products that are directly administered to the spot of the outer skin surface[3,4], where the drug molecules can penetrate through skin layers, producing non-systemic (e.g., local effects within skin layers) or, to a lesser extent, systemic effects (e.g., enter blood circulation)[5]. It is well known that topical administration can avoid the hepatic first-pass metabolism that could otherwise reduce drug absorption and bioavailability. Over the last decades, however, studies have revealed significant expression of skin enzymes in the metabolic activity of topical drugs[6-8]. These enzymes are described as drug-metabolizing enzymes (DMEs), which are capable of performing biotransformation and detoxification of drug molecules, and also potentially result in activation of toxic metabolites and cutaneous reactions[6,9].

Despite numerous topical products on the market, the enzymatic profile of DMEs and apprehension of other skin enzymes (e.g., which enzymes are present, where exactly they are localized, and the underlying biotransformation mechanisms) remain unclear. Assessing the roles of skin enzymes and downstream metabolic pathways following drug exposure is an essential approach for drug development and targeted drug delivery to ensure equivalent safety and efficacy in clinical translation [from *in vitro* data to *in vivo* or *in vitro-in vivo* correlation (IVIVC)]. Thus, this review aims to provide an overview of: (i) skin metabolic enzymes (DMEs), as well as their interactions with substrates and drugs, (ii) the role of epidermal enzymes in maintaining homeostasis, (iii) the evaluation of DMEs cellular locations, molecular functions, and their abundances using advance analytical and modeling techniques, (iv) drugs and vitamins metabolism in skin, and (v) key considerations in drug development. The work is expected to provide updates on skin DMEs and support the development of new research models, strategies and regulatory authorities toward the stage of standardized clinical practice and drug testing improvement.

SKIN MORPHOLOGY

The skin is composed of a multilayered structure, including the epidermis, dermis, and hypodermis, and features an intricate network of cells, nerves, and receptors. The epidermis, which is a thin stratified layer of epithelium with five different cell strata, is divided into two regions: the stratum corneum (SC) or non-viable epidermis, and viable epidermis consisting of stratum lucidum, stratum granulosum, stratum spinosum, and stratum basale[10]. Keratinocytes are the major cell type in the epidermis, making up 95% of the total, and play a vital role in sustaining the integrity of the skin barrier and homeostasis. More details of the roles of keratinocytes in homeostasis will be discussed in the following section. Other important cells such as Langerhans cells, melanocytes, and Merkel cells are distributed throughout the viable layers. Starting from the innermost layer of the epidermis, the stratum basale (SB) is anchored to the basement membrane, which is also known as the dermal-epidermal junction, by integrins. This layer consists of a large volume of highly proliferative cells, including the epidermal progenitor and stem cells. Continuous proliferation of these stem cells gives rise to specified cells such as melanocytes and, in particular, keratinocytes that can undergo stratification, differentiate and migrate upwards (from basale to corneum layer) to displace the surficial cells, a process called keratinization[11]. Several transcription factors (TFs), such as the activator protein-1 (AP-1), nuclear factor erythroid-related factor 2 (Nrf2), and aryl hydrocarbon receptor (AhR), are thought to regulate the signaling pathway of keratinocyte differentiation genes and govern the epithelial development[12]. Among the skin TFs, AhR is found to be expressed in every type of skin cell, including keratinocytes, melanocytes, fibroblasts, and immune cells, and it has been identified as a promiscuous binding site for many endogenous and exogenous compounds. Following the keratinocyte differentiation, the nuclei eventually degrade, and other organelles are catabolized by various epidermal enzymes[13]. Finally, the terminally differentiated cells form the outermost SC, which is made up of anucleated and flattened

dead keratinocytes (also termed corneocytes) embedded in the lipid matrix (such as ceramides, cholesterol, free fatty acids, and phospholipids). The arrangement of corneocytes in a dense lamellar structure in SC provides an efficient physical barrier of skin against the ingress of compounds from reaching viable tissue. The dermis, located underneath the SB layer, primarily consists of fibroblasts, which are responsible for the synthesis and secretion of collagen, elastin, and hyaluronic acid into the intercellular matrix. In the dermis, there are also other cells such as adipocytes, endothelial cells, and reticular fiber networks such as hair follicles, eccrine glands, and sebaceous glands. The hypodermis, the deepest layer of the skin, is composed of subcutaneous fat and loose connective tissue[14]. The compositions of skin with different stages of cell differentiation and expression reflect distinct barrier properties of the multilayered skin structure and, subsequently, the extent of metabolism.

DRUG-METABOLIZING ENZYMES

Once drugs pass the physical barrier, DMEs act as a biochemical barrier, metabolizing and detoxifying (or toxifying) drug compounds from skin cells[15]. DMEs are typically found in the viable epidermis, with the majority found in keratinocytes (the primary cells of the epidermis)[15-17]. These enzymes are classified into phase I (i.e., oxidation, reduction, hydrolysis) and phase II (i.e., conjugation)[18-20].

Phase I enzymes include cytochromes CYPs, alcohol dehydrogenases (ADHs), aldehyde dehydrogenase (ALDHs), aldehyde oxidases, amine oxidases (AOCs), carboxylesterase (CESs), cyclooxygenases, esterases, epoxide hydrolases (EPHX), leukotriene A4 hydrolases, and flavin-containing monooxygenases (FMOs)[8]. As one of the most versatile enzyme families, CYPs (especially CYP1-3) are responsible for > 75% of phase I metabolic activity of general drug types[19,21]. Along with the distinct classification of isozymes, most dermatological drugs are either substrates, inducers, or inhibitors of CYPs, resulting in a myriad of potential reactions (e.g., drug-drug interactions)[20]. CYPs and other enzymes from phase I facilitate detoxification through (i) the biotransformation of prodrugs to their active metabolites or by (ii) increasing the excretion rate that involves the conversion of lipophilic compounds into more hydrophilic compounds. Of importance, the former process has been associated with the potential generation of procarcinogens (which can then be metabolized into toxic carcinogens)[20,22]. On the other hand, the latter process may result in insufficient drug potency, leading to reduced therapeutic efficacy (or a low threshold for effectiveness). As a member of the cytochrome P450 group (phase I enzymes), CYP11A1 plays significant roles in the human skin, such as initiation of local steroidogenesis and metabolism of Vitamin D, as well as regulation of the protective barrier and skin immune functions. Skin pathology could happen with disturbances in CYP11A1 activity[23]. Action of CYP11A1 was observed on the unmodified side chain of Vitamin D3, which produces 20-hydroxyvitamin D3 [20(OH)D3] (considered as the major product), and further oxidizes the side chain to generate 20,23-dihydroxyvitamin D3 [20,23$(OH)_2$D3]; 20,22-dihydroxyvitamin D3 [20,22$(OH)_2$D3]; and 17,20-dihydroxyvitamin D3, with 20-hydroxyvitamin D3; 17,20,23-trihydroxyvitamin D3 [17,20,23$(OH)_3$ D3]; and 17-hydroxyvitamin D3 as minor products[24,25]. Other CYP enzymes such as CYP27A1, CYP2R1, CYP3A4, CYP24A1, and CYP27B1 could further metabolize the CYP11A1-derived hydroxyderivatives with the production of at least 18 hydroxy metabolites[25,26].

Phase II enzymes such as hydroxysteroid dehydrogenase (HSD), glutathione S-transferase (GST), UDP-glucuronosyltransferase (UGT), N-acetyltransferase (NAT), sulfotransferase, and acetyltransferase further promote the metabolism and elimination of drugs or intermediates through conjugation of more polar functional groups to the drugs. The GST is identified as the most prominent phase II enzyme, comprising three superfamilies (cytosolic, microsomal, mitochondrial)[27]. Altogether, the biotransformation of DMEs is characterized as the rate-determining steps in drug metabolism. Common DMEs found in human skin, including *ex vivo* native skin and reconstructed skin equivalents, are listed in Tables 1 and 2, though this list

Table 1. Common phase I drug metabolic enzymes detected in skin, its substrates or reactions performed, and topical drugs metabolized by the enzyme

Enzyme	Substrates/reactions performed	Topical drug	Reference
ADH1A	Aldophosphamide, aliphatic alcohols	Ziagen, cyclophosphamides	[28]
ADH1B	Aliphatic alcohols, hydroxysteroids, retinol	Retinyl palmitate	[28]
ADH1C*	Aliphatic alcohols, retinol, steroids	N/A	[28]
ADH5	Pentanol, ω-hydroxyl fatty acids	N/A	[28]
ALDH1A1	Acetaldehyde, aldophosphamide, aliphatic aldehydes, retinal	N/A	[29]
ALDH1B1	Acetaldehyde, aliphatic aldehydes, lipid peroxidation-derived aldehydes	N/A	[29]
ALDH1L1	10-formyl-tetrahydrofolate	N/A	[29]
ALDH2	Aldehydes	Nitro-glycerine	[29]
ALDH3A1	Medium-chain aliphatic and aromatic aldehydes	N/A	[29]
ALDH3A2	Fatty and long-chain aliphatic aldehydes	N/A	[29]
ALDH4A1	Glutamate-ϒ-semialdehyde	N/A	[29]
ALDH6A1	Methylmalonate semialdehyde, oxidative decarboxylation of methylmalonate semialdehyde to acetyl-CoA and propionyl-CoA	N/A	[30]
AO	Aza- (and oxa-) heterocycles, oxidize endogenous/exogenous aldehydes to carboxylic acid	Carbazeran, citalopram, pyridoxal, tamoxifen, vanillin, zoniporide	[31]
AKR1A1	Aldehyde	N/A	[32]
AKR1B1	Aldose	N/A	[32]
AKR1C1-3	Trans-dihydrodiols of aromatic hydrocarbons	N/A	[32]
AKR1D1	Δ4-3-ketosteroid 5β-stereospecific	N/A	[32]
AKR7A2	Azole (e.g., posaconazole, voriconazole)	Azole antifungal drug	[33,34]
CBR1	Aldehydes and ketones to hydroxyl	Laxoprofen, pentoxifylline	[35]
CYP1A1	Dibenzo [a, l] pyrene, 7,12-dimethybenz[α]anthracene	Dacarbazine	[36]
CYP1A2	Dibenzo [a, l] pyrene, 7,12-dimethybenz[α]anthracene	Acetaminophen, caffeine, imipramine, pirfenidone, theophylline	[36]
CYP1B1	Dibenzo [a, l] pyrene, 17β-estradiol, fatty acid, polycyclic aromatic hydrocarbons	Estradiol, melatonin, retinol	[37]
CYP2B6	Bupropion, O-deethylation of 7 ethoxy-4-trifluoromethyl coumarin	Aminopyrine, clonazepam, diazepam	[38]
CYP2E1	Chlorzoxazone hydroxylation, para-nitrophenol hydroxylase	Tretinoin	[39]
CYP3A4/5/7	Erytchromycin N-demethylase, cyclosporine	Bexarotene, benzodiazepines, clindamycin, lidocaine, tretinoin, opioids, sandimmune	[40]
CYP4B1	N-hydroxylation of aromatic amines, W-hydroxylation of lauric acid	N/A	[41]
CYP4F8	19-Hydroxylation of prostaglandin H1 and H2	N/A	[41]
CYP4F12	Oxidation of arachidonic acids into 18-hydroxy arachidonic acid	N/A	[41]
CYP7B1	25-hydroxycholesterol, 27-hydroxycholesterol, DHEA	Prasterone	[41]
CYP8A1	Eicosanoids	N/A	[8]
CYP11A1	Hydroxylation of cholesterol to pregnenolone, hydroxylation of Vitamin D3	Lanosterol, lumisterol 3	[26]
CYP2R1	25-hydroxylation of Vitamin D3	N/A	[42]
CYP24A1	Hydroxylation of 1α, 25(OH)$_2$D3 and 25(OH)D3	N/A	[42]
CYP26B1	Retinoic acid (Vitamin A)	Tretinoin	[43]
CYP27A1	27-hydroxylation of cholesterol and 25-hydroxylation Vitamin D3	N/A	[44]
CYP27B1	Vitamin D3 metabolism	N/A	[45]
CYP3A4 and CYP39A1	7α-hydroxylation of 24-hydroxy cholesterol	N/A	[41]
CYP51A1	4β-desmethyllanosterol, 24,25-dihydrolanosterol, 24-methylenedihydrolanosaterol, obtusifoliol	Imidazole	[46,47]

CYB5A	N/A	Ruxolitinib	[48]
FMO1	N-oxygenation of secondary and tertiary amines (benzydamine)	Benzydamine, ketoconazole, rifampicin	[49]
FMO3	Sulfides and tertiary amines to sulfoxide and N-oxide	Cimetidine, nicotine	[50]
FMO5	Ketones to esters	N/A	[50]
EPHX1 (dermis) and EPHX2 (epidermis)	Hydrolysis of arene and aliphatic epoxide to less reactive and become more water-soluble dihydrodiol by the addition of water (Trans)	N/A	[51]
STS (epidermis)	Hydrolysis of aryl and alkyl sulfate	Estrone sulfate, lovastatin	[52]

ADH: Alcohol dehydrogenase; *previously named as ADH1, ADH2, and ADH3; ALDH: aldehyde dehydrogenase; AO: aldehyde oxidase; AKR: aldo-keto reductase; CBR: carbonyl reductase; CYP: cytochrome P450; FMO: flavin-containing monooxygenases; EPHX: epoxide hydrolase; MHBD: 2-methyl-3-hydroxybutyryl-CoA dehydrogenase; STS: steroid sulfatase; DHEA: dehydroepiandrosterone; N/A: not applicable.

Table 2. Common phase II drug metabolic enzymes in skin, its substrates or reactions performed, with examples of inducers or inhibitors

Enzyme	Substrates/reactions performed	Topical drug	Reference
17β-HSD11	5-androstane-3,17-diol (3-diol) into androsterone	Glucocorticoids	[53]
GSTK1 (κ)	CDNB	N/A	[54]
GSTM2 (μ)	CDNB, glutathione	N/A	[55]
GSTO1 (Ω)	Ascorbic acid, CDNB	N/A	[56]
GSTP1 (π)	CDNB, ethacrynic acid glutathione	N/A	[57]
GSTT1 (θ)	CDNB S-hexylglutathione	N/A	[58]
MGST1	1-*S*-(glutathionyl)-2,4,6-trinitrocyclohexadienate; glutathione; glutathionyl-*S*-dinitrobenzene	N/A	[57]
SULT1A4	N/A	N/A	[59]
SULT2B1	DHEA, pregnenolone	Viramune	[59]
COMPT	Ascorbic acid, caffeic acid, daphnetin, flavonoids, gallic acid, green tea catechins, hydroxyquinoline	Benserazide	[60]
MPST	3-mercaptopyruvate (with dihydrolipoic acid and thioredoxin) to hydrogen sulfide	Hydrogen sulfide	[61]
TGM3	First undergoes proteolysis to its active form Acyl-transfer between glutamine and other primary amines essential for the formation of epithelial and hair follicle	N/A	[62]
TPMT	S-methylation of aromatic and heterocyclic sulphydryl	Azathioprine	[63]
TST	Thiosulfate	Hydrogen sulfide, sodium thiosulfate	[64]

HSD: Hydroxysteroid dehydrogenase; GSTK: glutathione-S-transferase; CDNB: 1-chloro-2,4-dinitrobenzene; SULT: sulfonyl transferase; COMPT: catechol-O-methyl transferase; MPST: 3-mercaptopyruvate sulfurtransferase; TGMs: transglutaminases; TPMT: thiopurine methyltransferase; TST: thiosulfate sulfurtransferase; DHEA: dehydroepiandrosterone; N/A: not applicable.

is not exhaustive.

IDENTIFICATION, CHARACTERIZATION AND ABUNDANCE OF SKIN ENZYMES

The advent of new approach methodologies (NAMs) such as omics- (e.g., metabolomics, genomics, proteomics and transcriptomics) and onics-based (e.g., electronics and photonics) tools have eased the bioprospection of a massive data for the enzymes, including the study of physicochemical properties and clinical information. Mass spectrometry (MS) is the most comprehensive proteomic tool and is often used in conjunction with other methods to manifest both qualitative and quantitative profiling at the single-cell level, as well as to discover novel enzymes.

Representing the most vital and largest interface between the body and surroundings, however, the skin is highly heterogeneous across intra- and inter-individual (or skin equivalents), resulting in various enzymatic activities and their cellular localization[16]. Kazem *et al.* have summarized the DMEs' activities in different skin equivalents[65]. Overall, mRNA expression of phase I enzymes is more plentiful than phase II[65,66]. The mRNA of CYP1A1 and CYP1B1 was found to be expressed in all tested skin models, including *ex vivo* native human skin (NHS). Specifically, both CYP1A1 and CYP1B1 are colocalized within the epidermis (matured keratinocytes), fibroblasts, and sebaceous gland, with little expression in sweat producing cells[67]. Dysregulation of CYP1A1 and CYP1B1 has been observed to mediate reactions that result in mutagenic and carcinogenic metabolites[20,68]. Their clinical implications should be cautiously scrutinized during drug development. Although mRNA expression was detected in other CYP subfamilies (e.g., CYP4, CYP7, CYP8, CYP11, CYP21, CYP26, CYP27, CYP39 and CYP51), they showed no significant cellular functionality[20,65,68].

The fact is that there is a low correlation between the levels of mRNA, protein expression, and molecular activities. A recent study performed by Couto *et al.* has observed and quantified 2,000 skin proteins using MS with label-free quantification (MaxQuant)[8]. Herein, instead of phase I, a higher phase II enzyme expression was reported at the proteomic level in both NHS and skin equivalents. GSTP1 (π) was the highest abundant (62.65 ± 17.78 pmol·mg^{-1}) phase II enzyme, followed by GSTM4 (μ) and GSTM3 (μ) at concentrations of 21.75 ± 0.63 pmol·mg^{-1} and 19.68 ± 10.29 pmol·mg^{-1}, respectively. This finding corresponds to the data reported by Götz *et al.*, Hewitt *et al.*, and Eijl *et al.*, where GSTP1 (π) protein (2-fold) and activity levels (8-fold) are higher in skin compared to liver[27,54,69]. The GSTP1 (π), in particular, has been demonstrated to play important roles in regulating epidermal cell growth and cellular apoptosis. Differentiated keratinocytes appeared to promote high expression of GSTP1 (π) and increase drug clearance[70]. In contrast to liver, UGT was not actively detected in any NHS and skin equivalents, suggesting that skin is a minor site for glucuronidation reaction[54]. Upon ligand binding to AhR, however, it may enhance UGT (UGT1A3) transcription in response to xenobiotic drug molecules. On the other hand, the most abundant enzymes from phase I are ADH1B, ADH1C, ALDH1A1, and carbonyl reductase (CBR), which are mainly found in the epidermis layers[71]. Among these enzymes, ADH1C is observed to be the highest expressed phase I enzyme (37.46 ± 15.05 pmol·mg^{-1}), yet the concentration is much lower as compared to GSTP1 (π).

Unexpectedly, CBR3, which was understood to be expressed highly in skin, was reported to have lower expression levels compared to CBR1, which is predominantly expressed in liver, kidney, and intestines. Only three CYPs enzymes, which are CYP8A1 (5.52 ± 4.70 pmol·mg^{-1}), CYP7B1 (2.57 ± 0.45 pmol·mg^{-1}), and CYP51A1 (0.98 ± 0.09 pmol·mg^{-1}), were detected. These CYPs enzymes are primarily involved in endogenous metabolism (i.e., cholesterol and eicosanoid metabolism). The result showed a controversy with the work of Kazem *et al.*, where no enzyme activity was assessed for the above CYPs enzymes[65]. Being the most abundant phase I DME in liver, CYP3A4 has low or no expression in all tested skin models[7,66]. Meanwhile, FMO1 and FMO5 were highly detected in engineered models, whereas FMO3 was only found expressed in NHS[7]. Other phase I enzymes have been detected, including AOs, AOCs, CES, and EPHX. In addition, Couto *et al.* have also identified and quantified redox enzymes, proteases, and nucleases[8]. Thioredoxin and peroxiredoxin 1 were reported to be the most abundant antioxidant enzymes in skin. Another study done by Liu *et al.* combined liquid chromatography with tandem MS (LC/MS/MS), non-invasive tap strips method, and the label-free MaxLFQ to quantify skin proteins in volunteers[72]. Herein, up to 1,157 epidermal proteins, including TGM1, TGM3, ALOX12B, and ALOXE3, were matched to the UniProt database[72]. As one of the most crucial enzymes in cornification, TGM was identified to be expressed in the granulosum and spinosum layers[73].

The epidermis was recognized as the major reservoir site for skin esterase, which is responsible for the conversion of methyl salicylate to salicylic acid[74,75]. Telaprolu *et al.*, instead, have identified greater metabolism in the dermis layer derived from the same donors, with greater area stained with esterase specific dye and a greater density, supporting higher dermal esterase prevalence[76]. The evidence further highlights the indispensable relationship between the biological complexity of skin models and enzyme activities. Nonetheless, the enzymes were mostly close or below the limit of detection[7]. The observed data can be explained by the discrepancy between the mRNA turnover rate and the protein translation level. Moreover, this can also be due to a limited metabolic pathway (i.e., a high saturation rate of metabolism). Skin metabolism is approximately only 10%-30% of that activity in liver, albeit most DMEs are detected in skin[16,65,77].

ROLE OF EPIDERMAL ENZYMES IN HOMEOSTASIS

The skin is continuously exposed to a number of endogenous and external compounds. In addition to serving as an effective barrier, it is capable of maintaining local and global homeostatic mechanisms (with respect to physical, chemical, immune, microbial and neuronal functional levels), i.e., pH, plasma concentration, and neuronal plasticity[78]. Apart from DMEs, there are other intra- and extracellular epidermal enzymes (EEs) responsible for regulating redox balance and dynamic equilibrium by enhancing the barrier structure functions[79,80]. As mentioned above, most enzymes are localized in the epidermal, and the driven biotransformation activity is substantially important in inducing the differentiation of organelle (i.e., melanin and keratin). For instance, TGMs mainly located in the spinosum and granulosum layers can synthesize and upregulate the expression of barrier-related proteins such as keratin, profilaggrin, loricrin and involurin, accelerating the keratinization and cornification. TGM-3, specifically, is a cross-linking protein for the formation of epidermal and reticular fibers[81]. Filaggrin is the major intermediate for the compaction of keratinocytes and the regulation of the epidermal terminal differentiation. During this process, the keratinocytes can modulate the expression and secretion of EEs, such as the kallikrein (KLK)-related proteases, enabling direct cleavage of extracellular and transmembrane epidermal proteins (e.g., corneodesmosin and desmocollin) in SC layer[80,82]. In healthy skin, desquamation and degradation of these proteins enable continual self-renewal of epidermal cells to maintain intact barrier homeostasis[82].

Moreover, essential lipids consisting of ceramides, cholesterol, and fatty acids (FA) are key components of the epidermis[78]. Common EEs involved in the lipids metabolism, that are important for keeping a functional skin barrier include β-glucocerebrosidase (converts glycosylceramides into ceramides), acidic sphingomyelinase (converts sphingomyelin into ceramides), and phospholipase A_2 (converts phospholipids to free FAs and glycerol). Another relevant aspect is the effect of pH, which is directly relevant for cell differentiation, formation of epidermal lipids (and other lipid compartments), maintenance of skin microbiome, and the promotion of suboptimal substrate turnover[83]. The epidermis has a low pH of 4.0-6.0. Both β-glucocerebrosidase and sphingomyelinase are reported to work optimally at pH 5.0-5.5[83]. The free FAs formed by phospholipid breakdown contribute to the acidic environment in SC, which is required for the regulating activity of many of the enzymes in SC and the barrier function. In reaction with the main stressors in skin, such as ultraviolet radiation (UVR), the phase 1-dependent skin cells and DMEs may generate carcinogenic stressors, including the reactive oxygen species (ROS) and nitric oxide (NO)[84]. The UVR can be classified into UV-A, UV-B, and UV-C, each with different electrophysical properties, and their penetration into skin is dependent on the wavelength. UV-A tends to penetrate to the innermost epidermis layer and to the dermis, while UV-B can be absorbed fully by the epidermis melanocytes[11]. The resulting radicals are responsible for the induction of inflammation cascade and degenerative photoaging[11]. These radicals can also alter nucleic acids (DNA and RNA) and protein expression, which result in mutations and are the leading factor in melanoma or non-melanoma cancers and drug

resistance[85,86]. To detoxify and eliminate these radicals, the skin is equipped with antioxidant EEs such as glutathione peroxidases (GPxs), superoxide dismutases (SODs), and catalases (CATs), which are expressed abundantly in the viable layers.

As a protective barrier between the internal human body and external environments, the sensory and adaptive capacity of skin could sustain local and global body homeostasis against harmful/unpleasant factors. The ability of skin to synthesize melatonin in response to external stress is critical and is considered one of its essential physiological functions[87]. Slominski *et al.* presented that melatonin (N-acetyl-5-methoxytryptamine) is a product of multistep tryptophan metabolism via serotonin and N-acetylserotonin[88]. Melatonin metabolism occurs rapidly through indolic and kynuric pathways caused by UVR or ROS. Antioxidative responses and DNA repair pathways can be stimulated by melatonin and metabolite products of melatonin. These properties of melatonin could be derived from the binding to quinone reductase 2 (NQO2), calmodulin (CaM) or the regulation of mitochondrial functions impacting cellular homeostasis. At pharmacological concentrations, melatonin can be used to counteract a number of damages caused by MT1- and MT2-independent mechanisms; this effect is mediated by the aryl hydrocarbon receptor (AhR), which is attributed to the structural similarity between melatonin and its natural ligands, such as tryptophan metabolites and indolic compounds[88].

In the research of Slominski *et al.*, UV energy can touch the central neuroendocrine system and reset the body homeostasis, including that of skin (cutaneous homeostasis)[89]. UV absorption by the skin triggers mechanisms defending skin integrity, and also causes skin pathology, such as cancer, aging, and autoimmune responses. Melanins, known as skin colors or biopolymeric pigments, are produced by melanocytes (which are specialized pigment cells)[90]. Melanins are also considered polymorphous and multifunctional biopolymers, represented by eumelanin, pheomelanin, neuromelanin, and mixed melanin pigment[89]. L-tyrosine and L-DOPA play significant roles as the substrates and intermediates of melanogenesis. They also act as positive regulators of melanogenesis and other cellular functions of the body, depending on internal and external factors[91]. The optical and chemical filtering properties of melanins, together with their cytoprotective ability, could help protect skin cells against environmental factors such as solar/UV radiation, free radicals, and toxic chemicals. Melanins achieve this by binding to these harmful substances and then transferring them into more stable complexes[90,92]. Nevertheless, according to Karkoszka *et al.*, owing to the formation of such complexes, locally and systemically administered drugs bound to melanins might not directly interact with receptors or enzymes.This could result in a lack of certain pharmacological and/or pharmacodynamic parameters, increasing the likelihood of side effects[92]. Additionally, there are risk factors for melanoma, deriving from the malignant transformation of melanin-producing melanocytes, which is a formidable malignancy[93].

Additionally, EEs are reported to be involved in hydration-induced changes (e.g., maturation of skin cells, desquamation process, and expression of protein) in different epidermal cells. For instance, a decrease in glycerol metabolism combined with reduced sebaceous gland activity leads to water loss in SC, causing an abnormal skin barrier and altered molecular mobility[94]. Several studies have also identified the role of EEs in antioxidant defense and redox metabolism; for instance, protein glutathione peroxidase 4 (GPX4) and thioredoxin reductase 1 (TXNRD1) are responsible for protecting skin against membrane lipid peroxidation[95].

METABOLISM OF DRUGS AND VITAMINS IN SKIN

Prodrugs and soft drugs

The entity of metabolites and the quantification of metabolomes have been a standard protocol to identify the amount of drugs permeated to different skin layers, and potentially predict the levels of metabolic activity. Prodrugs are intentionally designed to be inactive until they reach the site of action, in this case mainly within skin layers, and are converted to the active parent drugs by DMEs to exert pharmacological effects. These prodrugs can avoid extensive metabolism by skin enzymes, reduce off-target toxicity, and enhance the bioavailability of drugs at local sites. Telaprolu *et al.* have identified the presence of esterase enzymes in *ex vivo* human skin membranes by using methyl salicylate (in Metsal™ cream) as substrates[76]. Methyl salicylate is a widely available over-the-counter topical drug. It is a prodrug that is pharmacologically inert until it is metabolized into the therapeutically active salicylic acid (Aspirin) and methanol by the skin carboxylesterases in the viable epidermis and dermis layers [Figure 1]. The salicylic acid will be further metabolized (in liver) for clearance by phase II DMEs, i.e., CYP2C9, NAT2, and UGT1A6 through hydroxylation, acetylation, and glucuronidation, respectively[96].

Another example is tamoxifen, which is traditionally used as first-line treatment in breast cancer but has more recently been identified as a potential drug for exophytic dermatitis[97,98]. The parent drug is biotransformed by CYP2C9 to its potent form, 4-hydroxytamoxifen, with minimal off-target issues. Preformulation with nano-emulgel further increases its lipid solubility and enhances its topical SC penetration[97]. Hyaluronic acid (HA) is broadly incorporated in many cosmetics formulations to enhance skin rejuvenation and hydration, along with the capability to potentially support the wound healing process and prevent skin necrosis[99]. Topical HA, in its non-cross-linked form, however, is highly susceptible to enzymatic breakdown (e.g., hyaluronidase). Fortunately, the nano-prodrug formulation enhances its bioavailability by enabling controlled bioactivation and release upon reaching the target site, such as radicals production, overexpressed enzymes, and low pH intracellular environment[99]. Naturally-derived drug compounds (e.g. silibinin) exhibit low chemical stability and undergo rapid metabolism (mainly mediated by UGT1A1, UGT1A6, UGT1A9, GST and SULT), leading to rapid clearance from the body[100]. Silibinin is known for its photo-protective capabilities against UV-B to prevent hyperproliferation and differentiation of epidermal keratinocytes, and regulate inflammation signaling cascades[101]. Several research studies have been conducted to improve silibinin bioavailability through prodrug modification[102]. Silibinin loaded in hydrogel polymer with a combination of chitosan and fucoidan has been shown to favor longer drug retention time and skin distribution[103].

Contrary to prodrugs, soft drugs are therapeutically active molecules that undergo rapid systemic metabolism into inactive metabolites after exerting their local effects. The active drugs, thus, do not enter the systemic circulation and are promptly cleared from the body[104]. For example, crisaborole is a topical soft drug used to treat atopic dermatitis (AD). The drug is metabolized into 5-(4-cyanophenoxy)-2-hydroxyl benzylalcohol (AN7602) by CYP enzymes (e.g., CYP1A1/2 and CYP3A4) and hydrolases. It was then further metabolized to 5-(4-cyanophenoxy)-2-hydroxyl benzoic acid and AN7602-sulfate through oxidation and sulfation[105]. Both metabolites are inactive products lacking glucocorticoid activity[106]. Tom *et al.* have demonstrated relatively low levels of crisaborole topical ointment in human plasma, suggesting systemic detoxification through enzyme metabolism[106].

Vitamins

Vitamins (i.e., Vitamins A, C, D, and E) are actively used as important ingredients in many topical products. Endogenous retinoic acid (RA) is triggered during wound-induced hair follicle neogenesis (WIHN) and other skin damage responses (e.g., laser treatments)[107]. Bioactivation of Vitamin A (retinol) is through two-step oxidation, forming retinaldehyde and later to the biologically active RA by retinol

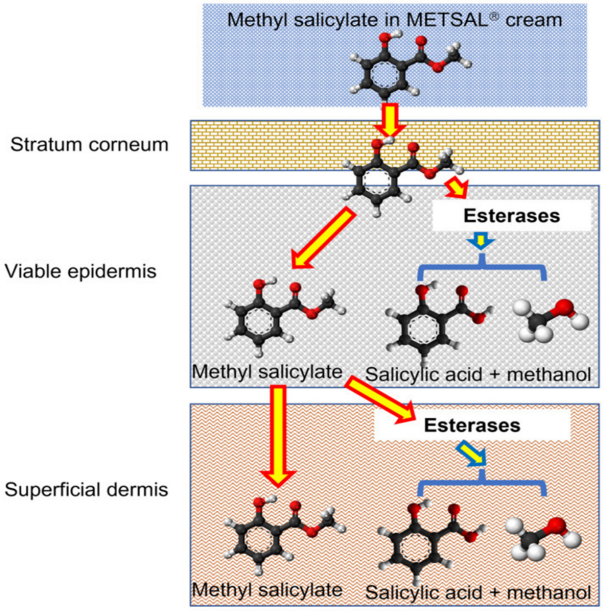

Figure 1. Metabolism of methyl salicylate by skin esterases. (Reproduced from "Human skin drug metabolism: relationships between methyl salicylate metabolism and esterase activities in ivpt skin membranes" by Krishna *et al.*, *Metabolites* 2023).

dehydrogenase (ALDH1 family) and CYP26 enzymes, respectively[108]. Retinol can also undergo esterification with fatty acid to form retinyl esters by retinol acyltransferase (LRAT) [Figure 2].

Synthesis of Vitamin D mainly occurs in the epidermis, where epidermal keratinocytes carry the most metabolic enzyme activity. Vitamin D3 is produced from 7-dehydrocholesterol (7-DHC) (photolysis) or derived from diet. CYP27A is reported to metabolize Vitamin D3 to 25OHD and further to its active metabolite 1,25$(OH)_2$D or 24,25$(OH)_2$D by CYP27B1 and CYP24A1, respectively[44,45].

DRUG DEVELOPMENT CONSIDERATIONS

The key considerations in topical drug development are to test whether the product is safe, potent and capable of reaching its desired clinical efficacy. Following the phase-out of animal trials in research, as mandated by FDA Modernization Act 2.0, a number of *in vitro* and *in vivo* skin permeation models, as well as NAMs, have been implemented to evaluate skin metabolism under controlled settings to allow for the optimization and delivery control[109]. Generic topical dermatological products can be safe, effective and affordable alternatives to branded medications. Yet, the complex nature of skin can impact skin enzyme expression and metabolic activity, causing high variability in the pharmacokinetics and pharmacodynamics (PK/PD) of the drugs[7]. Important scientific questions, thus, need to be addressed for drug assessment.

Roles of skin enzymes

The enzymes involved in the biotransformation of topically administered drugs, as well as their biotransformation mechanism and pathway, should be thoroughly investigated. This includes the reaction kinetics (Vmax/Km/Kcat/CLint), and toxicology profiles with potential induction and inhibition of enzymes. The enzyme saturation state is essential for determining the reaction rate and the amount of parent drugs available, especially when prodrugs or soft drugs are applied. Drug interaction following the enzyme biotransformation (or drug combinations) generates reactive metabolites that could potentially induce toxicity, causing pre- or post-marketing withdrawals. Although most DMEs in skin showed low

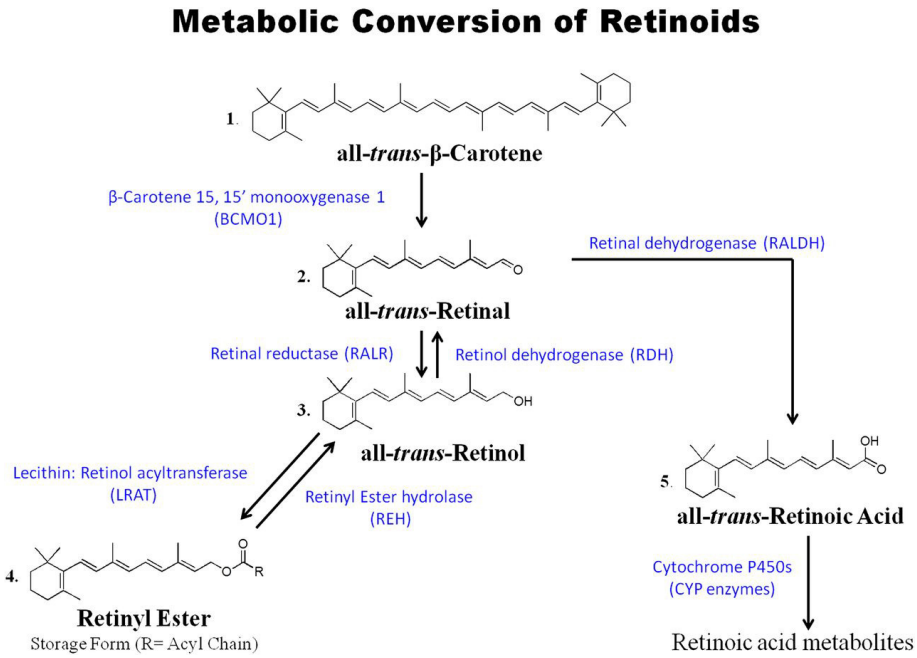

Figure 2. Enzyme-catalyzed Vitamin A metabolism. (Reproduced from "Retinol and retinyl esters: biochemistry and physiology" by Sheila M and William S, *J Lipid Res* 2013).

basal activity, leave-on topical products can have significant time-concentration effects following prolonged exposure.

Genetic variation

Intra- (e.g., transcriptomic heterogeneity across different skin regions) and inter- (e.g., age, gender, racial) individual differences can lead to case-to-case variation in drug activities.

Skin nature

The skin biological complexity, maturation stage, culture and isolation methods (from selection, preparation to storage conditions) can make huge disparities in drug permeation and enzyme expression. The reconstructed skin has an overall lower number of enzymes detected compared to *ex vivo* native human skin (NHS)[77]. The NHS and synthetic reconstructed skin comprise only an approximate 32% overlap in the identification and quantification of enzymes[8].

Correlation between mRNA-protein-cellular activities of enzymes

Levels of mRNA were shown to have poor proxies toward the quantification of protein and activity[69]. Plus, many DME activities are close to the limits of detection and quantification, which could reduce accuracy[8,69].

Skin equivalents

The skin models should have the ability to reflect *in vivo* conditions of drug metabolism. The selection, preparation, and isolation methods (e.g., heat and frozen treatments) are shown to affect enzyme turnover and metabolic activity[76].

There are also investigations regarding comorbidities associated with metabolic dysfunction that result in changes in DME expression and activities[110]. The alterations due to the disease state itself, as well as the treatments and comedication (e.g., drug- and/or enzyme-drug interactions)[110,111], can lead to a loss of

enzyme activity, causing the enzymes to fail to respond properly to the drugs. At the same time, many autoimmune and inflammatory-mediated reactions can also lead to abnormal mRNA and protein turnover. For instance, Sumantran *et al.* observed a meaningful reduction in DME levels in both melanomas and psoriasis[112]. Another study done by Candi *et al.* showed overexpression of TGM-5 in patients with ichthyosis vulgaris, while nearly absent in Darier's disease-affected skin[73]. Literature studies also suggested that various skin diseases can manifest the generation of free radicals (e.g., ROS) and trigger subsequent inflammatory responses, eventually resulting in the destruction of homeostasis and redox balance. Perihan *et al.* reported that in patients with acne vulgaris, there were significantly exacerbated levels of antioxidant EEs, as compared to healthy controls, due to the increase in oxidative stressors[113]. Conversely, the expression of EEs was found to decrease in severe acne than mild or acute conditions[113]. This could probably be attributed to the overproduction of ROS that leads to cellular senescence of enzyme-related proteins[95]. While evidence linking comorbidities status with enzyme expression, the exact underlying mechanism remains to be discovered.

Additionally, skin diseases could also alter the epidermal keratinocyte differentiation program, causing a defective skin barrier and affecting the delivery of topically administered drugs[95]. Psoriasis features hyperproliferation and upregulation of keratinocyte differentiation, resulting in abnormal protein metabolism and redox regulation. This disturbance contributes to the improper development of skin adhesion and junction proteins essential for intact barrier formation[114,115]. Meanwhile, the levels of filaggrin, together with the EEs involved in SC lipid biosynthesis, were significantly low in atopic dermatitis[116]. Alterations in the expression of barrier-related protein (or nucleic acids) are believed to contribute to epidermal cell degradation, increased lipids peroxidation and membrane permeability of topical drugs. Skin affected with vitiligo has selective loss of melanin resulting from autoimmune destruction of melanocytes in the epidermis and hair follicles. This disrupts the balance of the cutaneous antioxidant system, including both enzymatic and non-enzymatic molecules, inducing a pre-senescent status and compromised metabolic function[117]. Progressively, irregular expression of these enzymes and barrier-related proteins could result in skin barrier dysfunction, reduced drug biotransformation, and eventually result in excessive drug accumulation at local sites or in systemic circulation.

Hence, it is always important to understand the comprehensive role of enzymes in skin to ensure the safety and efficacy of the topically administered drugs. Information on metabolizing enzymes in skin is also highly helpful in assessing and comprehending the bioequivalence of topical products in terms of qualitative (Q1) and quantitative (Q2) composition, as well as physical and microstructural arrangement of matter (Q3). Even if Q1/Q2 similarity is achieved, the grade of excipients and enhancers needs to be considered, as they can have a significant impact on enzyme metabolism and final drug product quality attributes.

CONCLUSION

Numerous works have demonstrated the presence of DMEs with significant skin metabolizing activity. Although enzyme expression (both transcriptional and translational levels) and activity in the skin are generally much lower than in the liver, the skin is still the largest interface between the body and surroundings; hence, the skin's net capacity for metabolic processes and other cellular effects can be considerable. Owing to their ability to induce biotransformation and bioactivation, these DMEs can bring both positive and adverse impacts. A comprehensive understanding of these enzymes could provide a basis for promising *in vitro* to *in vivo* extrapolation, including dose optimization and targeted drug delivery. However, there are no verified and validated standard protocols for quantification parameters of enzymes in the skin compartment, hampering the efficiency and reliability of research findings, thereby reducing the accuracy of assessment tools. Furthermore, the complexity of skin morphology, the scarcity of studies on

the relationship between skin enzymes and topical drugs, and the low correlation among different types of data derived from mRNA, protein, and activity studies pose further challenges in drug development. Given these issues, future research shall continue to fill the gaps relating to complications of metabolic profiling, improve data incorporation into modeling systems, and refine existing predictive frameworks.

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Authors' contributions

Writing - original draft preparation: Lau N
Writing - review and editing: Phan K
Supervision, revisions and proofing: Mohammed Y

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The authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

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Consent for publication

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