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ORIGINAL RESEARCH

Chlormethine Gel for the Treatment of Mycosis Fungoides Cutaneous T-Cell Lymphoma: In Vitro Release and Permeation Testing

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ABSTRACT

Introduction: The DNA-alkylating agent chlormethine (CL, or mechlorethamine) is approved in several countries worldwide as a 0.016% w/w topical CL gel formulation, to treat mycosis fungoides cutaneous T-cell lymphoma, with a positive benefit/risk ratio.

Methods: Release profiles of CL from the gel and a compounded ointment-based 0.016% CL formulation were compared via in vitro release testing (IVRT), utilizing static diffusion cells, a pseudo-infinite dose, and polytetrafluoroethylene membranes, over 5 h. The percutaneous absorption profile of CL gel in ex vivo human skin was also examined, using in vitro permeation testing (IVPT) with flow-through diffusion cells, dermatomed skin (epidermis plus dermis) and epidermal membranes, a finite dose, over 24 h.

Results: In IVRT experiments, the mean ± SD CL release rate was significantly higher for the gel versus the ointment (5.70 ± 0.73 versus 2.38 ± 1.03 $\mu g/cm^2/\sqrt{h}$); the formulations were inequivalent per the US Food and Drug Administration scale-up and postapproval changes for nonsterile semisolid dosage forms (FDA SUPAC-SS) criteria. Mean IVPT cumulative CL (gel) permeating through epidermal membrane was higher than for dermatomed skin (4.6% versus 2.5% of applied dose). Mean residual CL on the epidermal membrane surface was 1.3% of the applied dose.

Conclusions: CL gel (0.016%) and ointment were inequivalent, with an optimized release profile, suggesting minimal passage of CL gel through human epidermal tissue to the dermis.

Keywords: Chlormethine gel; In vitro release; In vitro permeation; Mycosis fungoides

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Key Summary Points

Why carry out this study?

Cutaneous T-cell lymphoma is a group of non-Hodgkin's lymphoma, of which mycosis fungoides is the most common.

Chlormethine is an alkylating agent recommended by international guidelines as a first treatment option for adults with mycosis fungoides.

What did the study ask?/What was the hypothesis of the study?

In a preliminary, proof-of-concept, in vitro release testing (IVRT) study, we probed the equivalence of the in vitro release profiles of 0.016% chlormethine from the registered gel formulation (reference) and from a compounded ointment-based formulation (Aquaphor); the percutaneous absorption profile of chlormethine gel in ex vivo human skin was also evaluated using in vitro permeation testing (IVPT).

What was learned from the study?

The rate of chlormethine release from the gel formulation was significantly greater than from its ointment-based counterpart.

There was minimal passage of chlormethine through the epidermis in human skin samples, suggesting that most of the applied chlormethine reacted within that layer (i.e., before reaching the dermis), where it would likely exert its effects in mycosis fungoides-lesioned skin.

INTRODUCTION

Mycosis fungoides (MF) is a primary cutaneous T-cell lymphoma (CTCL), characterized by the infiltration of malignant T-cells to the epidermal layer of the skin [1]. MF accounts for ≥ 60% of CTCLs and ≥ 50% of all cutaneous lymphomas [2]. Although the prognosis for early-stage disease can be acceptable (5- and 10-year survival rates > 90%), a quarter of patients are at risk of progression to advanced disease, which has a poor prognosis (survival < 4 years) [1, 3, 4]. There is no cure for MF-CTCL with conventional systemic therapy; available therapeutic options focus on local treatment of lesions (for all stages), preventing disease progression (mainly in late stages), and maintaining patient quality of life (QoL) [1, 5]. Skin-directed therapies (SDTs) are the foundation of care for early-stage disease [6, 7], and palliation at all stages, with adjunctive therapy for treatment and symptom management in more advanced MF-CTCL [5, 8].

Chlormethine (CL; also known as mechlorethamine) is a bifunctional deoxyribonucleic acid (DNA)-alkylating agent that inhibits rapidly proliferating cells [1, 9, 10]. CL also induces DNA double-stranded breaks, increases caspase 3 gene expression, and suppresses DNA-repair genes in a subpopulation of malignant skin T-cells from patients with MF-CTCL [11]. CL is a well-established and effective topical therapy for MF-CTCL [12, 13]. After clinical administration, CL is rapidly converted to its active form (ethylene immonium ion), and is usually cleared from the blood within a few minutes. Applied topically, CL is rapidly metabolized via hydrolysis and demethylation, typically within minutes of making contact with body constituents such as water [14–17]. Consistent with these properties, pharmacokinetic analyses indicate no evidence of systemic absorption of CL following topical application of either gel or ointment formulations [18–20]. In a subgroup of patients enrolled in Study 201, CL gel (0.02%) was administered once daily, per protocol, for up to 12 months. There was no measurable evidence of systemic absorption, assessed in blood samples taken up to 1 month after the first application. Patients who did not achieve a complete response in Study 201 participated in the subsequent open-label extension, Study 202, in which they received 0.04% CL gel daily for a further 7 months—again with no measurable evidence of systemic exposure for up to 6 months after the first application

[20]. CL is not stable in aqueous or ointment formulations; however, 0.016% CL gel ("CL gel") has a nonaqueous vehicle with butylhydroxytoluene (promoting stability) and diethylene glycol monoethyl ether (which can form an intracutaneous depot when administered topically, thus reducing possible systemic CL absorption) [12, 14, 21]. It is the first SDT gel formulation to be purposely developed to treat MF-CTCL [12, 17, 22, 23]. CL gel has demonstrated noninferiority compared with the original ointment (Aquaphor Healing Ointment, Beiersdorf Inc, Hamburg, Germany) [24]. CL gel response rates were consistently higher than the CL ointment for the primary endpoint of Composite Assessment of Index Lesion Severity, in both intent-to-treat and efficacy-evaluable populations [24]. In additional studies, CL gel contributed to improved QoL in patients with MF-CTCL, with a favorable benefit/risk ratio and no evidence of systemic absorption [17, 20, 23, 24]. Moreover, the physical properties of CL gel—stable, nongreasy, and quick drying—render it easier to apply and more adherent to the administration site, increasing the likelihood of patient compliance [12, 25]. CL gel is approved in Europe for first-line treatment of adult patients with MF-CTCL, and in the USA to treat adult patients with stage IA/IB MF-CTCL who have received prior SDT [26, 27]. It is now available commercially in several countries worldwide, and guidelines for the treatment of MF-CTCL consistently recommend topical CL as a first-line SDT [5–7].

A preliminary, proof-of-concept, in vitro release testing (IVRT) study compared the release profiles of 0.016% CL from CL gel and a compounded ointment-based formulation (Aquaphor); in vitro permeation testing (IVPT) was also conducted to evaluate the percutaneous absorption profile of CL gel in ex vivo human skin.

METHODS

The aims of this study were twofold: (1) assess for equivalence the in vitro release profiles of CL from the registered gel formulation (reference) and from a compounded ointment-based formulation (test) across polytetrafluoroethylene (PTFE) membranes (determined using IVRT), using US Food and Drug Administration (FDA) scale-up and post-approval changes for nonsterile semisolid dosage forms (SUPAC-SS) guidelines where appropriate [28], and supportive statistical analyses (*t*-test assuming unequal variances); (2) evaluate CL permeation from the registered 0.016% gel formulation across ex vivo human skin using IVPT.

The suitability of the synthetic membrane (IVRT), receptor solutions (IVRT, IVPT), and extraction fluid (IVPT) used in these experiments was established in separate experiments, as described in Supplementary Appendix 1 and 2.

Formulations

CL gel 0.016% w/w (Valchlor [mechlorethamine] or Ledaga [chlormethine]) was shipped and stored frozen (ca. −20 °C) until use; it was used as received (commercial 60 g tube) for in vitro experiments. Appropriate laboratory safety measures were used when handling CL. The compounded, ointment-based formulation was prepared in-house by first dissolving CL to 1.6% w/w in neat ethanol (Acros Organics, Morris Plains, NJ, USA). An aliquot of the ethanolic solution was subsequently diluted into Aquaphor and mixed vigorously until homogenous, yielding a CL strength of 0.016% w/w. This compounded ointment-based formulation was prepared immediately before use and discarded following the experiment.

IVRT

Experimental setup

Glass vertical diffusion cells (VDCs) (Fig. 1a) (Southeastern Laboratory Apparatus, North Augusta, SC, USA) with an open-top donor compartment (dosing area, ~ 2 cm^2) and ~ 10 mL receptor compartment were used. Dosing areas and receptor volumes were measured for each individual cell and measured values were used in subsequent calculations (see below). The PTFE membrane (3 M, Maplewood, MN, USA) was sandwiched between the donor

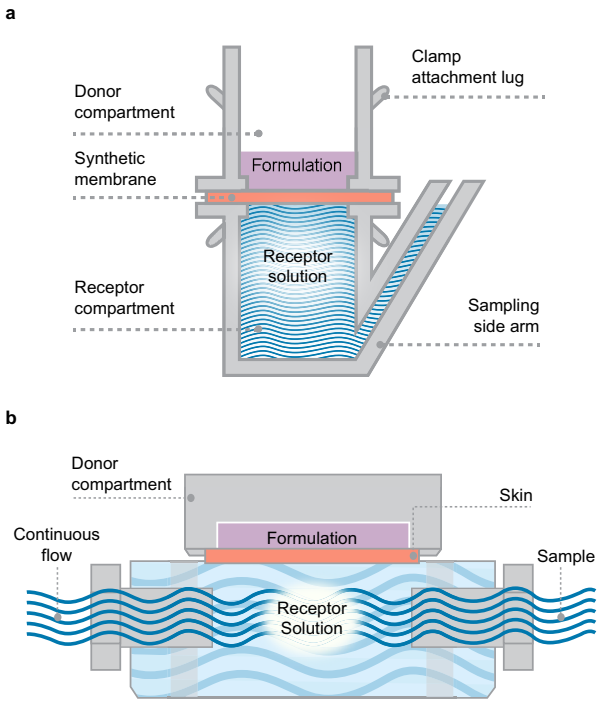

Fig. 1 Experimental setups. In vitro release testing (**a**) and in vitro permeation testing (**b**)

and receptor compartments. The receptor compartment was filled with a receptor solution comprising propylene glycol: isopropyl alcohol: glycerol (40:40:20) with 0.1% formic acid, 0.01% butylated hydroxytoluene, and 0.01% sodium chloride. After allowing equilibration of the assembled VDC to a membrane surface temperature of 32 °C, 300 mg portions of each formulation were dispensed evenly onto the PTFE membrane. Receptor solution samples (0.75 mL) were collected at time (t) = 0 and regularly thereafter for up to 24 h (fresh aliquots of receptor solution were added to the receptor chamber after each sampling to return the volume to 10 mL).

Sample analysis

The receptor solution samples were chemically derivatized (in a fume hood) using a modification of the procedure described by Cummings et al. [29]. CL concentrations in these derivatized samples were measured using ultra-high-performance liquid chromatography (UHPLC) (Nexera 3-Series, Shimadzu, Columbia, MD, USA) coupled with an ultraviolet (UV) detector. The samples were injected on to a Waters Acquity CSH C18 column (2.1 × 50 mm, 1.7 μm particles) and eluted with a linear gradient of acetonitrile (mobile phase B) and 5 mM phosphoric acid in water, pH 3 (mobile phase A). The gradient program was 25% B from 0 to 0.25 min, a gradient from 25% B to 95% B from 0.25 to 1.75 min, and a hold at 95% B until 2.25 min. The flow rate was 0.65 mL/min, the column was held at 40 °C, the samples were held in a chilled autosampler at 10 °C during analysis, the injection volume was 1 μL, and detection was performed at 276 nm using a diode array UV/visible light detector (Shimadzu). Receptor solution samples were quantitated against a calibration curve (0.05–5.00 μg/mL). Use of independently prepared quality-control samples confirmed the acceptable accuracy (± 5%) and precision (≤ 5%) of the method.

Calculations

Rates of CL release over time were calculated from the slopes of plots of the cumulative amount of CL released per dosing area (μg/cm^2) versus the square root of time ($\sqrt{}$h). Statistical comparisons of the reference and test formulations were made on six replicates from the full-scale experiments based on FDA SUPAC-SS guidelines, whereby equivalence requires a 90% confidence interval (CI) of 75–133% [28], and using a t-test assuming unequal variances.

Small- and full-scale experiments

Initially, a small-scale experiment (n = 3 replicates) was performed to determine the optimal time points and temperature for measuring the release of CL in subsequent full-scale experiments (n = 6 replicates). Small-scale experiments were run at 32 and 25 ± 1 °C; the receptor solution was sampled at time (t) = 0, 0.5, 1 h, and hourly thereafter for 7 h, then at 24 h after application of the CL formulations.

IVPT

Experimental setup

Healthy, top-layer, barrier-competent skin harvested from elective abdominal surgeries was used, either separated into epidermal membranes or dermatomed (epidermis plus dermis) to a thickness of 500 μm (full-thickness skin ~ 2000 μm). Skin samples were mounted into the donor compartment of separate flow-through diffusion cells designed to mimic physiologic/anatomical skin in situ (Fig. 1b). The receptor solution that flowed continuously underneath the skin samples was citrate phosphate buffer pH 4.0 with 30 mM NaCl + 0.1% lactic acid. CL gel was applied to the upper surface of the skin at a dose of 10 mg/cm^2, covering an area of 1 cm^2. Blank experiments were run without application of the CL gel.

Samples of the receptor solution were collected automatically every 2 h post-dose for 24 h. At the end of each run, residual CL gel was cleaned from the skin surface using cotton swabs and a single tape strip, and extracted from the cleaning materials into a solution of 100% acetonitrile (extraction fluid).

Sample analysis

Concentrations of CL in receptor solution and surface wash samples were measured using UHPLC (Waters Acquity I-Class) coupled with a triple-quadrupole mass spectrometer (MS/MS, Waters Xevo TQ-XS, Milford, MA, USA) following the same chemical derivatization procedure used for the IVRT experiments. The UHPLC column was a Supelco Titan C18 (2.1 × 20 mm, 1.9 μm particles) and CL was eluted with a linear gradient of methanol (mobile phase B) in 5 mM ammonium formate with 0.1% formic acid in water (mobile phase A). The gradient program was 5% B from 0 to 0.25 min, a gradient from 5% to 95% from 0.25 to 2.25 min, and a hold at 95% B until 2.50 min. The flow rate was 0.5 mL/min, the column was held at 30 °C, the samples were held in a chilled autosampler at 10 °C during analysis, and the injection volume was typically 10 μL. The MS/MS was operated in positive ion, multiple reaction monitoring mode, and monitored the 382.0/176.0 (Q1/Q3) transition for derivatized CL with a collision energy of 20 eV, capillary voltage of 3 kV, a cone voltage of 80 V, a source temperature of 150 °C, and a desolvation temperature of 400 °C. Samples were quantitated against a calibration curve (1.02–100 ng/mL). Independently prepared quality control samples were used to confirm the acceptable accuracy (± 20%) and precision (≤ 20%) of the method.

Calculations

The following parameters were calculated: cumulative amount of CL that permeated into the receptor solution over time (ng/cm^2), cumulative amount of CL at the final time point (ng/cm^2), residual CL on the skin surface at the conclusion of the run (ng), and flux rates (ng/cm^2/h). Outliers were rejected according to internal procedures.

Compliance with Ethics Guidelines

The human materials/samples used in this study were harvested in accordance with the principles of the 1964 Declaration of Helsinki and its later amendments [30] following receipt of the donors' informed consent and approval from an institutional review board (Pearl IRB, Indianapolis, IN, USA; IRB Study Number: 15-MEDP-101; Study Title: Healthy volunteer skin donation for in vitro experimentation). All donors provided informed consent to participate in the study.

RESULTS

In vitro release testing

A small-scale IVRT experiment evaluated the optimal temperature and data-collection times for the subsequent larger-scale experiments. In both experiments, the test agents (CL gel and a compounded ointment-based 0.016% CL formulation) were applied to a PTFE membrane mounted in a VDC (Fig. 1a).

Table 1 Rate of CL release from gel and ointment formulations across a PTFE membrane

Formulation	Rate of CL release[a] (slope; μg/cm^2/$\sqrt{h}$)		Linearity (r^2)		
	n	Mean ± SD	*n*	Mean ± SD	CV of release rate (%)
Small-scale experiments					
Gel[b]					
32 °C	3	6.41 ± 0.47	3	0.99 ± 0.00	–
Ambient (25 °C)	3	2.49 ± 0.39	3	0.95 ± 0.02	–
Ointment (Ambient)	3	0.75 ± 0.33	3	0.96 ± 0.02	–
Full-scale experiments					
Gel	6	5.70 ± 0.73	6	1.00 ± 0.00	12.80
Ointment	6	2.38 ± 1.03	6	0.99 ± 0.01	43.36

CL chlormethine, *CV* coefficient of variation, *IVRT* in vitro release testing, *PTFE* polytetrafluoroethylene, *SD* standard deviation

[a]The rate of CL release from the two formulations was calculated from the amount of CL in the receptor solution (propylene glycol: isopropyl alcohol: glycerol [40:40:20] with 0.1% formic acid, 0.01% butylated hydroxytoluene, and 0.01% sodium chloride) measured between 0.5 and 7 h or 5 h after application in the small- and full-scale experiments, respectively

[b]The small-scale IVRT for the gel formulation (reference) was assessed at both temperatures to determine the optimal temperature for assessing release rate

Full-scale experiments

Based on the small-scale experiment findings (Table 1, Fig. 2a; electronic Supplementary Appendix Results), full-scale experiments were conducted at 32 °C, and the receptor solution was sampled 0.5–5 h after application of the CL gel and ointment formulations to the PTFE membrane. The rate of CL release was significantly higher from the gel than from its ointment-based counterpart over the 5 h data collection period, at (mean ± SD) 5.70 ± 0.73 μg/cm^2/$\sqrt{h}$ (coefficient of variation [CV]: 12.80) and 2.38 ± 1.03 μg/cm^2/$\sqrt{h}$ (CV: 43.36), respectively (Table 1; Fig. 2b). The 90% CI on the ratio of mean rates of release was 25–59%; therefore, per the comparison methodology provided in the US FDA guidelines for SUPAC-SS [28], the formulations were considered inequivalent. This finding was supported by additional statistical analysis (*t*-test assuming unequal variances), demonstrating that both the rate and cumulative amount of CL released were significantly greater from CL gel than from its ointment-based counterpart (both $P < 0.0001$) (electronic Supplementary Appendix; Fig. S1; Tables S1 and S2).

In vitro permeation testing

Epidermal membrane from three donors ($n = 7$ replicates each) and dermatomed skin (epidermis plus dermis) from a single donor ($n = 7$ replicates) mounted into the donor compartment of flow-through diffusion cells were used in the IVPT experiments (Fig. 1b).

Permeation and penetration of CL through ex vivo human skin

The amount of CL in the receptor solution rose from 2 h and plateaued ~ 8–10 h after CL gel application for both the epidermal membrane and dermatomed skin (Fig. 3a and b). The mean cumulative amount of CL in the receptor solution after 24 h was 73.3 ng/cm^2 for the epidermal membrane and 40.4 ng/cm^2 for the

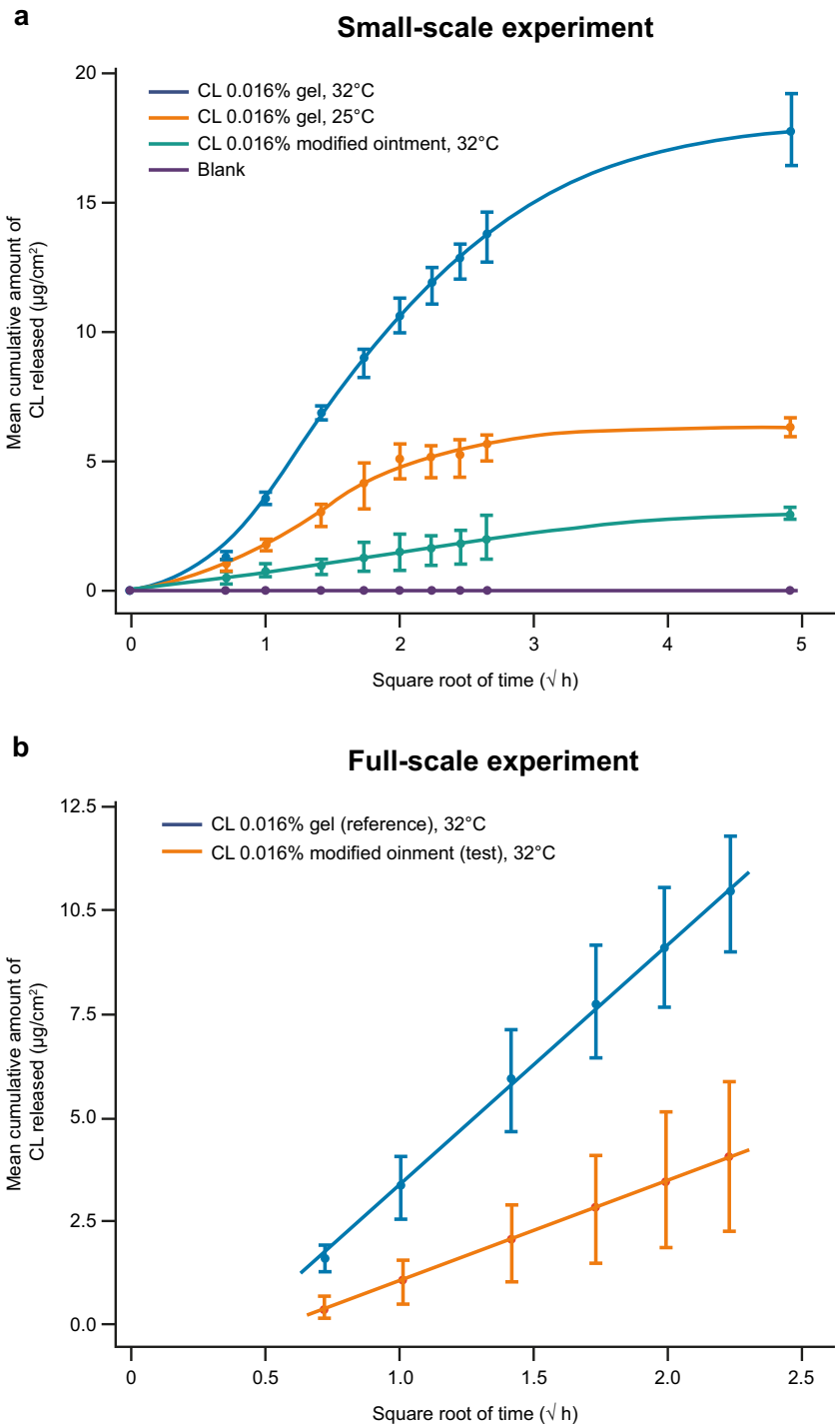

Fig. 2 Release of CL from gel and ointment formulations. **a** Small-scale and **b** full-scale experiments. Small-scale experiments were conducted first to determine the optimal temperature and sampling times for assessing CL release from gel (reference) and modified ointment (test) formulations across a PTFE membrane. The data are presented as **a** mean (range) cumulative amount of CL released per unit area (µg/cm^2) between 0 and 24 h after application (n = 3) and **b** mean (SD) cumulative amount of CL released from the reference and test formulations at 32 °C, over a 5 h experimental period (n = 6). *CL* chlormethine, *PTFE* polytetrafluoroethylene, *SD* standard deviation

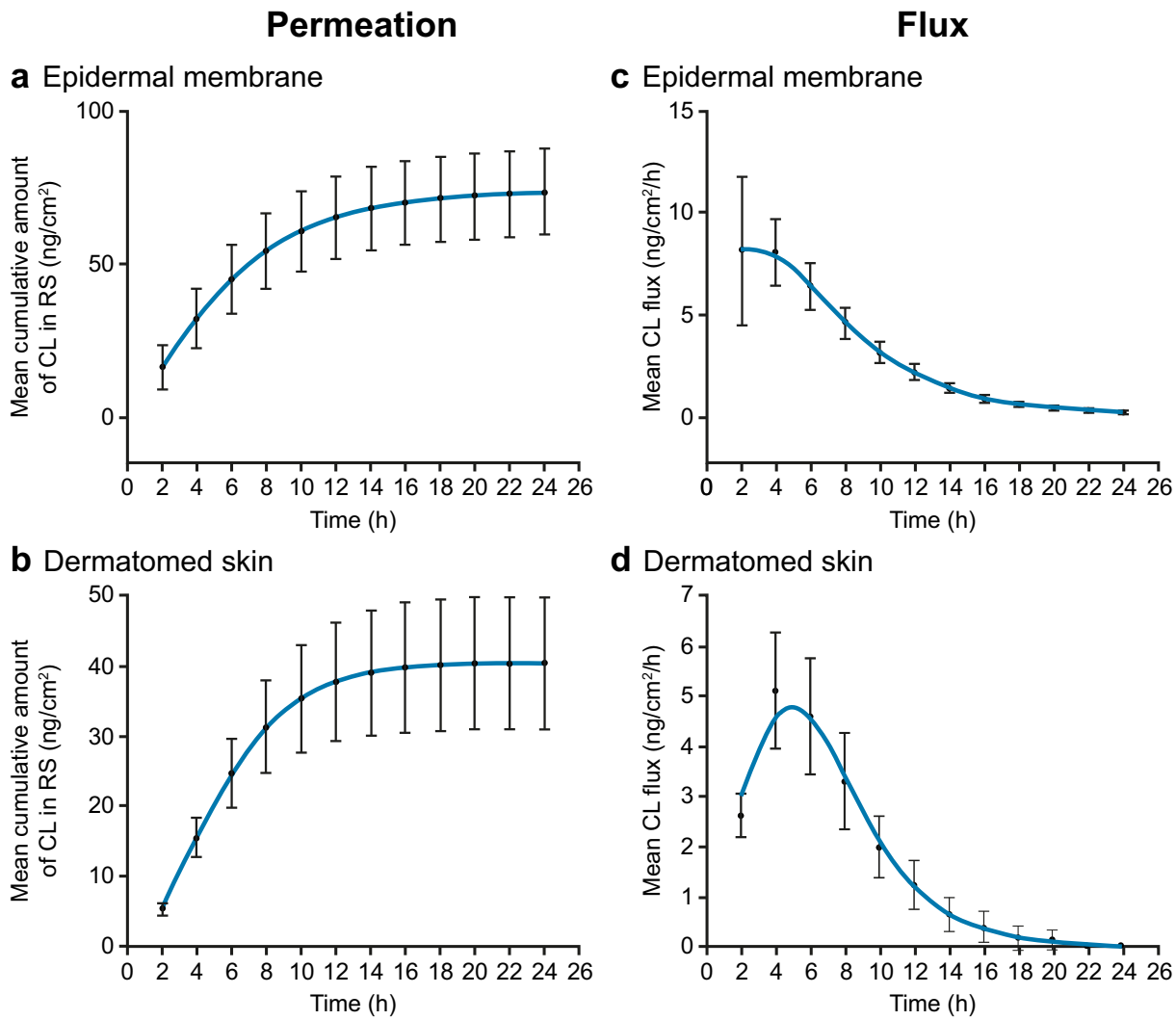

Fig. 3 Permeation and penetration of CL through ex vivo human skin. In vitro (**a**, **b**) cumulative amount and (**c**, **d**) flux of CL through epidermal membrane (n = 3 donors, 19 replicates[a]) and dermatomed skin (n = 1 donor, 6 replicates[b]). Epidermal membrane data are presented as mean ± SE; dermatomed skin is mean ± SD. [a]Two replicates were excluded from the analyses due to failure of receptor solution collection. [b]One replicate with an abnormal permeation profile was removed. *CL* chlormethine, *RS* receptor solution, *SD* standard deviation, *SE* standard error

dermatomed skin, representing 4.6% and 2.5% of the applied doses, respectively (Table 2). Flux of CL across the skin samples peaked ~ 2 h after application for epidermal membrane and ~ 4 h for dermatomed skin, with mean peak CL flux values of 10.8 and 5.2 ng/cm^2/h, respectively (Table 2; Fig. 3c and d).

Residual CL on the skin surface

Mean residual CL retrieved from the skin surface of epidermal membranes at the end of the experiment (24 h after application) was 21.0 ng (1.3% of the applied dose) (Table 2). Levels of CL on the surface of dermatomed skin were undetectable; 55.4 ng of CL was detected on the blank membrane, likely due to contamination.

Table 2 Cumulative amount and flux of CL, and residual CL on the skin surface 24 h after CL gel application

Skin sample	Cumulative amount permeated in RS (ng/cm^2)	Applied dose permeated into RS (%)	Peak CL flux into RS ($ng/cm^2/h$)	Residual CL on skin surface (ng)	Residual CL on skin surface (% of applied dose)
Epidermal membrane (mean ± SE)					
0.016% CL gel	73.3 ± 14.1[b]	4.6 ± 0.9[b]	10.8 ± 3.6[b]	21.0 ± 4.7[c]	1.3 ± 0.3[c]
Blank[a]	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0		
Dermatomed skin (mean ± SD)					
0.016% CL gel	40.4 ± 9.4[d]	2.5 ± 0.5[d]	5.2 ± 1.1[d]	BLQ	BLQ
Blank[a]	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0		

BLQ below the limit of quantification, *CL* chlormethine, *RS* receptor solution, *SD* standard deviation, *SE* standard error
[a]Experiment run with no CL formulation applied
[b]Three donors, 19 replicates; receptor solution was not collected for 2 replicates, which were therefore excluded from the analysis
[c] Three donors, 12 replicates; only 12 of the possible 21 replicates had quantifiable residual CL on the membrane surface
[d]One donor, 6 replicates; 1 replicate with an abnormal permeation profile was removed

Quantification of residual CL on the skin surface according to individual donors is provided in Table S3.

DISCUSSION

IVRT experiments comparing the rates of CL release from the gel formulation and a compounded ointment-based formulation revealed their inequivalence: the rate of CL release from the gel was significantly greater than from its ointment-based counterpart (5.70 versus 2.38 $\mu g/cm^2/\sqrt{h}$). IVPT experiments demonstrated that the percutaneous permeation profiles of CL from the gel formulation were numerically greater in epidermal membrane than in dermatomed skin, which could be expected given the lack of dermis in the epidermal membrane, and that CL had passed in small amounts through the epidermal tissue to reach the dermis. The metabolism of CL has not been fully elucidated; however, its rapid chemical transformation (rate unknown) after contact with body constituents [15, 17] may explain the small amount of CL able to pass unaltered through the epidermis.

Among the numerous SDT options, some are not approved for MF-CTCL, and others are burdensome or have limited efficacy. Treatments may be selected according to patient characteristics, likely side effects, and patient preference [1, 5, 8]. The CL gel data currently available—including efficacy, lack of systemic absorption, and our finding that most of the CL in the applied gel seems to act in the epidermal layer—suggest that this agent is a potentially effective treatment option for MF-CTCL. Indeed, the possibility that CL from the gel formulation acts predominantly in the epidermis, which is the focus of the skin infiltration of malignant cells in MF-CTCL [1, 31], may explain its observed efficacy [23, 24], suggesting that it could be most effective in early stages of the disease. However, further study is needed to confirm this hypothesis. Of interest, given that in MF atypical cells are located predominantly within the epidermis [1], a recent in vitro study confirmed that CL directly inhibits mainly

rapidly proliferating malignant T-cells in MF [11].

Real-world studies indicate that CL gel is efficacious for skin lesions in patients at all MF-CTCL stages [12, 23, 32]. CL gel is also an improvement on ointment-based formulations: its non-greasy, quick-drying properties makes it easier and more convenient to use, it has higher response rates in patients with stage IA–IIA MF-CTCL [23], and it is associated with improved patient QoL [23]. Regarding safety, the original registration trial for CL gel found no serious adverse events associated with the gel formulation, with a favorable benefit/risk ratio for the treatment of MF-CTCL [24]. Moreover, there were no signs of hematologic or systemic toxicity and no evidence of systemic absorption [20]. Consistent with those findings, the present IVPT data revealed negligible permeation of CL through dermatomed skin or epidermis. Extended exposure to CL via the 0.04% gel formulation after treatment with 0.02% CL gel conferred further improvement without increased toxicity [22]. Overall, the data suggest that CL gel with optimized drug release is crucial for treatment efficacy and patient compliance.

CL gel is approved for the treatment of MF by the FDA and the European Medicines Agency (in several countries worldwide); its preparation must therefore be compliant with current Good Manufacturing Practice, and subject to stringent safety, quality, and efficacy evaluations [33–35]. Despite the existence of an approved drug, some patients still seek—and in many countries some physicians still prescribe—a compounded form of CL, predominantly as an aqueous solution or ointment. Extemporaneously compounded formulations are not appropriately assessed for quality, stability, or efficacy [36–38]. The compounding process, including the ingredients, carries risks such as unknown stability, nonhomogeneous mixing of the active ingredient and base, degradation of the active ingredient (formulation failure), and introduction of a contaminant that could spoil the formulation or spread infection. These factors may compromise treatment efficacy, and often necessitate a short expiry date [21, 39–41]. There remains a place for compounded medicines, including for patients with conditions for which there is no commercially available or FDA-approved option; otherwise, use of such formulations confers unnecessary risk [37, 40].

Some study limitations should be acknowledged. There may be differences in structure between the nonstandard, extemporaneous, compounded ointment-based and gel formulations (e.g., particle size) that could impact their release profiles across a PTFE membrane. Variability in tissue structure between donors at different sampling sites may have impacted the findings; there are no relevant data on skin biopsy samples. While this is minimized by the use of healthy, human, lesion-free skin samples, it should be noted that the data from dermatomed skin were obtained using samples from a single donor. The reactivity of CL renders it impossible to measure its concentration in skin tissues (epidermis and dermis); however, this can be extrapolated by measuring the difference in quantities found in the collection buffer and from any residue of the applied dose. Finally, further experiments are needed to ascertain the final location of the CL following topical application.

In conclusion, the rate of CL release was significantly higher from the CL gel formulation than from the compounded ointment-based formulation, potentially correlating with the higher response rate observed with the gel versus the same ointment-based formulation during the noninferiority pivotal registration trial (NCT00168064) [24]. Based on these data, the FDA SUPAC-SS guidelines methodology, and additional supporting statistical analyses, the two formulations were determined to be inequivalent. The high variability observed in the IVRT data from the ointment-based formulation (Table 1) reflects the challenge of generating consistent, homogeneous ointment-based formulations. Regarding permeation profiles, there was a trend toward greater CL delivery and flux through the epidermal membrane compared with dermatomed skin. The data suggest that only a small portion of the applied CL dose can pass through epidermal tissue to reach the dermis, with an even smaller portion passing through the dermis of dermatomed skin. These

findings align with the lack of clinical evidence to support systemic exposure to CL [20, 24], effectively excluding the occurrence of significant absorption through the skin. Given the proportions of applied CL that passed through the skin (to the receptor solution) and remained on the skin surface, it is possible that most of the applied CL reacted within the epidermal layer (i.e., before reaching the dermis), where in MF-lesioned tissue it would exert its clinical efficacy.

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Author Contributions. Conceptualization: CG, SF, AA, VS, PLO-R. Formal analysis: PLO-R. Methodology: PLO-R. Project administration: CG, SF, AA, VS. Supervision: CG, SF, AA, VS. Writing – reviewing and editing: CG, SF, AA, VS, PLO-R.

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Disclosures. Claudio Giuliano, Stefano Frizzarin, Alessandro Alonzi and Virginia Stimamiglio are employees of Helsinn Healthcare SA. Pablo L. Ortiz-Romero has served on advisory boards for 4SC, Actelion, Helsinn, Innate Pharma, Kyowa, MYRAGEN, Recordati Rare Diseases, and Takeda; has a patent for PLCG1; and has received research support from MEDA.

Compliance with Ethics Guidelines. The human materials/samples used in this study were harvested in accordance with the principles of the 1964 Declaration of Helsinki and its later amendments [30] following receipt of the donors' informed consent and approval from an institutional review board (Pearl IRB, Indianapolis, IN, USA; IRB Study Number: 15-MEDP-101; Study Title: Healthy volunteer skin donation for in vitro experimentation). All donors provided informed consent to participate in the study.

Data Availability. The data that support the findings of this study are available from the corresponding author upon reasonable request.

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