Formulation, characterization and *in vitro* evaluation of antifungal activity of Nystatin microemulsion for topical application

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Abstract: The current research aims at development and assessment of o/w nystatin microemulsion. The pseudoternary phase diagrams were developed to determine microemulsion existence regions by water titration method. Nystatin was liquefied in the blend of oil phase, surfactant and cosurfactant. Microemulsion was made by deliberate mixing of water and stirring in this blend. The S-mix (surfactant-cosurfactant mixtures) of the ratio 1:2 was found better than 1:1 and 2:1 S-mix ratios. In vitro permeation studies by Franz diffusion cell revealed faster rate of nystatin release from such microemulsion (5.37µg/cm^2/h) as compared to nystrin (4.79µg/cm^2/h), a commercially available aqueous suspension. Kinetic modeling demonstrated zero order drug release and release mechanism found to be anomalous i.e. superposition of dispersion and swelling controlled drug release. Antifungal activity was performed using well diffusion method *in vitro* against *Candida albicans* cultures grown on Sabouraud's dextrose agar. The results also confirmed the high diffusion rate of drug from microemulsion as compared to aqueous suspension. The outcomes of this study propose that topical microemulsion of nystatin provides better antifungal activity as compared to emulsion gels or aqueous suspensions.

Keywords: Microemulsion, nystatin, permeation studies, flux, *candida albicans.*

INTRODUCTION

Microemulsions are widely employed in pharmaceutical research as they are elegant, easy to apply on skin, thermodynamically stable, feasible for large scale manufacture and improve the efficacy of hydrophobic drug (Date and Nagarsenker, 2008). Microemulsions have been previously prepared for topical administration as they can incorporate large amounts of lipophilic drugs as compared to the conventional creams and gels (Kumar *et al.*, 2010, Lawrence and Rees, 2012). Larger amounts of topically applied agents can be delivered through microemulsions into the mucosa than the traditional creams and gels (Kumar *et al.*, 2010, Güngör *et al.*, 2013). Microemulsions are amongst novel colloidal drug carriers to make sure dermal administration of antifungals by dermal targeting (Güngör *et al.*, 2013). While microemulsion can be used to deliver drugs through numerous routes; the system has been extensively studied as vehicle for topical administration. Microemulsions have gained much interest in pharmaceutical research due to their advantages such as ability to improve efficacy of hydrophobic drugs, thermodynamic stability, ease of manufacture and scale up (Date and Nagarsenker, 2008, Shah *et al.*, 2009).

Nystatin is an antifungal drug secluded from *Streptomyces noursei*. It is used for the management of skin, vaginal, mouth and intestinal tract infections (Pappas *et al.*, 2015). Parenteral administration of nystatin is prohibited due to severe systemic side effects and it is currently marketed for topical and oral routes of administration. Due to its lipophilic nature, nystatin has been formulated as lipophilic drug delivery systems (Samein, 2014). Nystatin suspensions for oral administration have been widely reported to exert gastrointestinal side effects. Thus, topical formulations of nystatin are preferred due to few side effects of skin such as hypersensitivity, rash, itching and burning which are often tolerable (Stefanovic *et al.*, 2013). Topical dosage forms are widely accepted by patients due to noninvasive nature and ease of self-administration (Güngör *et al.*, 2013). However, formulation of topical nystatin is challenging due to low stability and aqueous solubility. Microemulsions have been previously prepared for topical administration as they can incorporate large amounts of lipophilic drugs as compared to the conventional creams and gels (Kumar *et al.*, 2010). The current study aimed to develop and characterize nystatin microemulsion for topical delivery. Microemulsion was compared with a commercially available aqueous suspension of nystatin (Nystrin) for the antifungal activity on fungus culture. *In vitro* release kinetics of newly developed formulation also compared with the available conventional formulation of Nystatin.

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MATERIALS AND METHODS

Materials

Nystatin (Harmann Pharmaceuticals Limited Lahore, Pakistan), Tween 20 & 80 (Mac & Rains Pharmaceuticals, Lahore, Pakistan), Oleic acid (Simagchem Corporation, P.R. China) and Propylene glycol (Atlas Chemical Industries Lahore, Pakistan). All other chemicals were of analytical reagent mark.

Selection of excipients for nystatin micro emulsion

Due to having highest solubility of nystatin in them; Oleic acid (as oil phase), Tween® 20 (as surfactant) and propylene glycol (PG) (as Co-surfactant) designated as excipients for further studies.

Pseudoternary phase diagrams construction

Oleic acid, Tween® 20 and PG were titrated at 25°C contrary to H_2O. Tween® 20 and PG were blended in diverse concentration fractions 1:1, 1:2 and 2:1 and each surfactant-cosurfactant combination was blended with oil in different concentration ratios. The fraction of oil to the blend of surfactants and cosurfactants extended from 1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2 and 9:1 (w/w). Then those compositions of components were noted at which isotropically clear formulations were obtained. The transparent phase thus formed were indication of stable micro emulsions (Luo *et al.*, 2011, Moghimipour *et al.*, 2012).

Formulation of nystatin micro emulsions

Nystatin (0.4% w/w) micro emulsion were articulated after creation of pseudoternary phase diagrams, by mingling different concentrations of oleic acid, Tween 20, and PG under constant stirring (table 1). After 30 minutes, an isotropically clear, transparent nystatin microemulsion was instinctively formed.

Characterization of microemulsion

Centrifugation

The microemulsions were centrifuged to check out the stability of the selected formulations.10 ml of each microemulsion was taken in centrifuge tubes and then centrifuged for 30 min at 10000 rpm. Phase separation after centrifugation is an indicator of instability.

Visual appearance and clarity

The appearance and clarity of microemulsions can be determined by visual inspection of microemulsion formulations (Mandal and Mandal, 2011).

Determination of pH

The pH of the skin lies between 4 and 6 and all topical formulations must reside within that pH range. During the shelf life of product, changes in pH value may also be an indication of stability problems.

Conductivity measurements

The electrical conductivity properties of microemulsions were determined with conductometer (InoLab Cond 7110, WTW) at ambient temperature (25°C) (Mandal and Mandal, 2011).

Refractive index measurements

Refractometry measures the extent to which light is refracted when it moves from air to a microemulsion in order to assess their composition or purity. One drop of each microemulsion formulation was placed on the slide and refractive index was measured by digital refractometer (RX-5000, ATAGO Co, Ltd., Japan).

Determination of droplet size

Malvern Zetasizer was used for droplet size determination. Small amount of filtered deionised water was added to a glass cuvette and then little amount of diluted sample was added to that cuvette which was then placed between the detector and the laser light. Readings were taken for size and polydispersibility.

Viscosity measurements

The change in viscosity of the product gives an indication of stability changes. The viscosity values of microemulsions were obtained at 25°C at different rotation rates (rpm). Viscosities of formulations F1 to F5 were measured using spindle 2 at 10, 20, 30, 50, 60, 100 rpm (Brookfield viscometer RV DV-II + Pro model).

Release studies

Franz Diffusion Cell was used to determine in vitro release rate of formulations. The six Franz cells with volume of each about 10 ml (at 32˚C and 600rpm) and an area of about 0.95 cm^2 were used with a hydrophobic (nylon) membrane. The receptor media consisted of methanol and dimethyl sulphoxide (DMSO). Samples of 1 ml were taken at predetermined rate and analyzed by UV-Visible spectrophotometer at 308 nm after dilution with methanol and DMSO mixture.

Permeation data analysis

$J = K_p * C$

Where

Kp (Permeability coefficient) = DK/h

Kp and flux (J) was calculated as µg/cm^2 /h (Malakar *et al.*, 2012, Jain *et al.*, 2005).

Kinetic modeling of nystatin release profiles was done by fitting dissolution data in various models. Order of drug release was determined by zero and first order model whereas mechanism of drug release was measured by Higuchi, Hixon-Crowell and Korsmeyer-Peppas model.

In vitro assessment of antifungal activity

In vitro assessment of antifungal activity was done by measuring zone of inhibition (ZOI). ZOI was measured

on agar medium containing Sabouraud dextrose agar powder (65mg) dissolved in distilled water (1L). The isolates of 3µl/ml from standard *Candida albicans* suspension (10^7cells/ml) were seeded in three separate flasks containing agar medium. These inoculated agar mediums were poured in petri plates (15cm diameter) up to 4-5mm depth. Wells were made in each plate using Wassermann tube and sealed with molten dextrose agar (Marzouk *et al.*, 2012). Antifungal activity of final microemulsion formulation equivalent to 40 mg nystatin as well as 1gm Nystrin, a marketed suspension containing 100,000 IU/mlof nystatin were measured by well diffusion method. Measured quantity of each formulation was taken in separate *Candida albicans* inoculated plate and incubated for 24 hrs. Zone of inhibitions were measured in millimeter for each plate. This experiment was repeated three times and means values were compared antifungal activity (Ellaithy and El-Shaboury, 2002).

RESULTS

Solubility of nystatin in different oils

Solubility in IPM, oleic acid and liquid paraffin was found to be 194.5, 980.8 and 920.53µg/ml respectively.

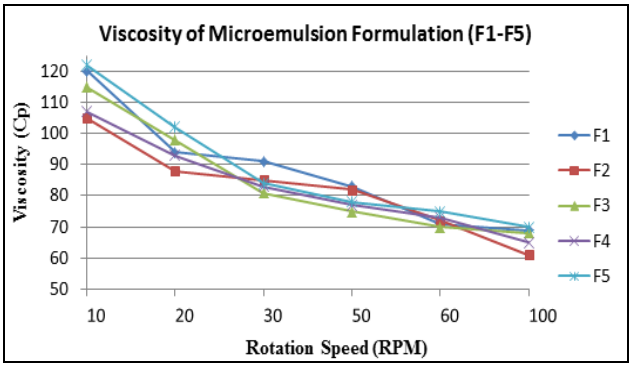

Fig. 1: Viscosity (cp) of Microemulsions (F1-F5)

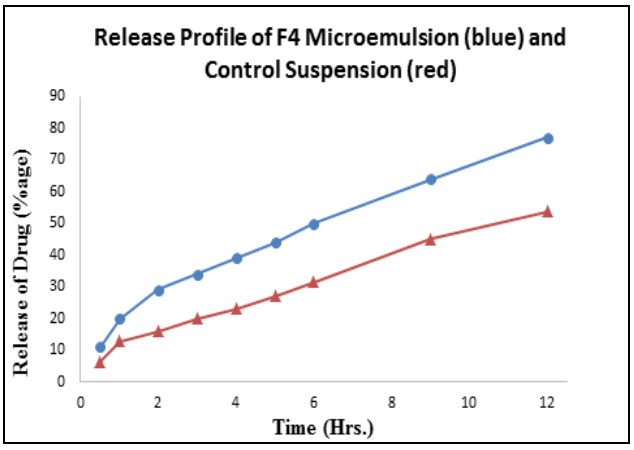

Fig. 2: Comparison of Mean Percent Amount Released from F4 Microemulsion and Control

Solubility of nystatin in different surfactants

The solubility of nystatin in Tween® 20, Tween® 80 and Span®20 was calculated. Its highest solubility found in Tween®20 which was 26.94±0.02 µg/ml.

Solubility of nystatin in different Co-Surfactants

Its highest solubility was seen in Propylene glycol (13.43 ±0.015) and least in n-butanol (0.48±0.110) among tested cosurfactants. Thus, PG was chosen as co surfactant due to highest solubility of nystatin among tested co-surfactants.

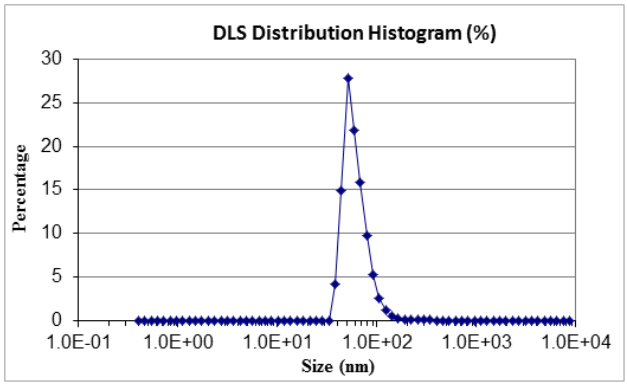

Fig. B1: Distribution histogram of F4 microemulsion

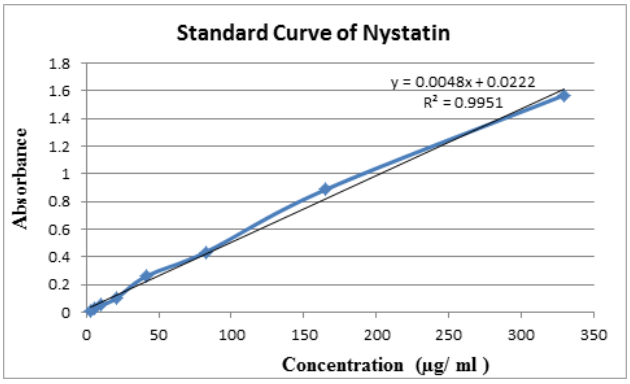

Fig. B2: Standard curve of nystatin

Pseudoternary phase diagrams

Diagrams containing Tween 20 to PG ratio 1:1, 1:2 and 2:1 were constructed to determine the microemulsion existence region in each diagram. Then, after construction of the pseudo ternary diagram the nystatin microemulsions were formulated by water titration method.

Characterization of micro emulsion

Evaluation of Organoleptic Parameters

Organoleptic parameters including appearance and clarity of all microemulsions (F1-F9) were determined. The F1-F8 microemulsions were observed to be yellowish and clear but F9 microemulsion became turbid upon storing and showed separation of two phases. Thus, F9 was excluded from further studies.

Table 1: Composition (w/w) of different nystatin microemulsion formulations with S-Mix of 1:2

Formulation code	Oil	Surfactant	Co-surfactant	Water	Nystatin
F1	5.94 %	17.8%	35.6%	40.38%	0.24%
F2	18.28%	24.7%	48.45%	8.22%	0.37%
F3	28.06%	22.45%	43%	6.08%	0.37%
F4	37.8%	18.89%	37.8%	5.19%	0.38%
F5	47.44%	16.13%	31.31%	4.7%	0.38%
F6	57.47%	12.45%	25.86%	3.83%	0.38%
F7	67.37%	9.62%	19.25%	3.37%	0.38%
F8	77.4%	6.48%	12.86%	2.9%	0.39%
F9	87.5%	3.21%	6.51%	2.43%	0.39%

Table 2: Evaluation of pH, conductivity and refractive index of different microemulsion formulations (Mean Values ± S.D.)

Formulation	pH	Conductivity (µS/cm)	Refractive Index
F1	5.67 ± 0.005	27.2 ± 0.10	1.416 ± 0.0005
F2	5.33 ± 0.010	5.60 ± 0.11	1.441 ± 0.0006
F3	5.29 ± 0.005	4.03 ± 0.05	1.445 ± 0.0002
F4	5.14 ± 0.017	2.80 ± 0.05	1.446 ± 0.0005
F5	4.92 ± 0.005	1.30 ± 0.06	1.449 ± 0.0002
F6	4.72 ± 0.011	1.03 ± 0.05	1.449 ± 0.0006
F7	4.55 ± 0.01	0.83 ± 0.06	1.453 ± 0.0005
F8	4.4 ± 0.011	0.33 ± 0.05	1.456 ± 0.0006

Centrifugation

Centrifugation at high speed can be used to predict mechanical stability of microemulsions. F1-F8 microemulsions were subjected to centrifugation and no phase separation or precipitation was observed by centrifugation at 3500 revolutions per minute (rpm) for 15 minutes (Min.).

pH

The pH values of all microemulsions (F1-F8) varied from 5.67 to 4.4 (table 2) that are within the acceptable range for skin preparations.

Conductivity

F1 microemulsion containing highest water contents showed highest conductivity values of 27.2µS/cm. As the water contents decreased, conductivity values also decreased (table 2).

Refractive index

The refractive index measurements for all tested microemulsions were found in desired range (1.416-1.456) for transparent isotropic systems (table 2).

Viscosity

Viscosity measurements are important to evaluate influence of on drug product performance, shelf life and ease of application. Viscosities of F1- F5 microemulsion formulations were measured using Brookfield viscometer (fig. 1).

Drug content

F4 microemulsion's drug content (99.24%) was highest of all formulations (table 3.). F4 microemulsion showed highest entrapment efficiency. F4 microemulsion and was proceeded for in vitro comparison of drug release and antifungal activity with commercially marketed aqueous suspension (as control).

Droplet size determination

Droplet size of F4 micro emulsion formulation was 51 nm as revealed by dynamic light scattering studies.

Table 3: Drug content of microemulsions (F1-F5)

Microemulsion Formulations	Content of Drug (%)
F1	96.35%±0.02
F2	95.12%±0.12
F3	97.14%±0.05
F4	99.24%±0.02
F5	96.34%±0.02

In vitro nystatin release studies and calculation of flux

The drug release studies were determined for F4 microemulsion and nystatin aqueous suspension (control formulation) using Franz diffusion cell (fig. 2). Percentage release of F4 microemulsion was found to be higher (77%) as compared to control formulation (54%).

Assessment of antifungal property

F4 Microemulsion showed greater zone of inhibition than marketed suspension whereas placebo microemulsion showed no antifungal activity (table 4).

Table 4: Antifungal activity of microemulsion, emulsion gel, aqueous suspension and placebo microemulsion.

Serial Number	Control	Microemulsion	Placebo Microemulsion
1	3	3.7	0.1
2	2.7	3.4	0
3	2.5	3.4	0
Mean	2.73	3.5	0.08
Standard Deviation	± 0.251	± 0.173	± 0.057

DISCUSSION

Due to having high solubility value, oleic acid was selected as oil phase for microemulsions to ensure high entrapment efficiency due to highest solubility of nystatin in selected formulation (Zhang and Ma, 2013, Zhang *et al.*, 2013). Tween 20 was selected as surfactant due to highest solubility of nystatin among tested surfactants. PG has high polarity which could enhance the solubilization properties of oleic acid (Kumar *et al.*, 2012). In addition, it has been previously used for fungicide (Olitzky, 1965), humectant and cleansing properties in different cosmetic and dermatological preparations. The transparent region in pseudo ternary phase diagram represented the micro emulsion existence region in phase diagram (Mandal and Mandal, 2011).

Table A1: Solubility of nystatin in different oils

Oil	Solubility (µb/ml)
Olive oil	0
Sesame oil	0
Isopropyl myristate	194.5
Liquid paraffin oil	920.5
Oleic acid	980

Table A2: Droplet size (nm) distribution of F4 microemulsion

Size Classes (nm)	Distribution Data (%)
32.67441559	0
37.83956146	4.2454772
43.82120895	14.90045166
50.74842834	27.7857399
58.77069855	21.78446579
68.06112671	15.88238811
78.82017517	9.724177361
91.28000641	5.275101185
105.7094727	2.629354

The microemulsions remained clear and transparent after centrifugation which confirmed mechanical stability of microemulsion (Karamustafa and Çelebi, 2008). All topical formulations must comply with normal skin pH range (pH 4-6) to avoid skin irritation. A change in pH of formulation may an indicator of instability during shelf life of product and may also influence the solubility of drug in the formulation(Mandal and Mandal, 2011). The o/w micro emulsions show low refractive index as compare to w/o microemulsions due to low refractive index of water (1.3336±0.0004) as external phase. The refractive index values were found to increase by increasing oil content in microemulsion due to relatively high refractive index of oleic acid (Kumar *et al.*, 2012). Higher electric conductivity is an indicator of o/w emulsion. Water as continuous phase would allow electric current to pass easily as compared to water droplets in dispersed phase. Microemulsions F6, F7 and F8 showed relatively lower values of conductivity which may be due to structural transition (Georges and Chen, 1986) and low water contents. Thus, formulations F1, F2, F3, F4 and F5 were preceded for further studies. Viscosity of all formulation decreased with increasing rotation per minute (rpm) which displayed non-Newtonian and pseudoplastic flow features which is extremely desirable for topical microemulsions. These microemulsions are stable yet ensure easy application to the skin. Reduction in the viscosity may also enhance absorption of nystatin after topical application (Hathout and Nasr, 2013). The viscosity values of F1-F5 microemulsion ranged from 105cp-122cp at 10 RPM which were constantly decreasing with increasing RPM. The mean droplet diameter of microemulsions is an important parameter that affects physical stability (Tenjarla, 1999). The small average droplet diameter obtained of F4 microemulsion was due to the penetration of propylene glycol (co-surfactant) molecules into Tween 20 (Surfactant) film around the oil droplets which reduced the fluidity and surface tension of the interfacial film (Chin *et al.*, 2014).

Higher release rate from microemulsion can be ascribed to greater solubility of nystatin in oleic acid (oil phase) compared to aqueous suspension as well as small particle size. F4 microemulsion has smallest droplet size of 58nm leading to higher drug incorporation and also increased drug release. In addition, surfactants and cosurfactants in microemulsions exert penetration enhancing effect by facilitated diffusion due to shuffling of lipid (80%) and aqueous (15-20%) layers in the skin (Kumar *et al.*, 2012). Flux values of F4 microemulsion, and aqueous suspension (control) are 5.37, and 4.79µg/cm^2/h, respectively. Higher flux and permeation coefficient values predict higher diffusion rate across a membrane. Results of this study suggested that nystatin will be delivered more efficiently across skin by micro emulsions than aqueous suspensions (Üstündag-Okur *et al.*, 2014).

Table A3: Kinetic modeling of *in vitro* release data

Formulations	Zero Order	First Order	Higuchi Model	Hixson Crowell Model	Korsmeyer-Peppas Plot	
	R^2	R^2	R^2	R^2	R^2	*N*
F4 Microemulsion	0.993	0.853	0.963	0.984	0.974	0.561
Control Suspension	0.994	0.819	0.961	0.99	0.984	0.683

Table A4: Changes in pH values of F4 microemulsion at 4°C, 25°C, 40°C, 40°C + 75 % RH during stability studies

Time	4°C	25°C	40°C	40°C + 75 % RH
Fresh	5.15	5.15	5.15	5.15
24 hrs	5.15	5.15	5.14	5.16
48 hrs	5.14	5.15	5.12	5.15
72 hrs	5.14	5.14	5.11	5.11
1st Week	5.13	5.12	4.98	4.96
2nd Week	5.12	5.11	4.81	4.84
3rd Week	5.08	5.01	4.7	4.73
4th Week	5.01	4.98	4.65	4.71

In kinetic modeling studies, rate and contrivance of drug release were estimated by putting release rate data of F4 microemulsion and control into different release models (table 3.). R^2 values of zero order model (0.993 and 0.994), Higuchi (0.963 and 0.961) and Hixson Crowell models (0.984 and 0.990) were obtained for microemulsion and control, respectively. Release exponent (n) in Korsmeyer-Peppas model for microemulsion (0.561) lied between 0.5 to 0.9 which predicted that drug was released by anomalous transport i.e. superposition of diffusion and swelling controlled drug release (Barzegar-Jalali *et al.*, 2008).

The antifungal property of advertised nystatin suspension (control), nystatin microemulsion and placebo microemulsion were revealed against (*Candida albicans* and zones of inhibition were compared (5). Higher antifungal activity of nystatin microemulsion compared to aqueous suspension is due to higher solubility of nystatin in oleic acid. Solubilized nystatin in oleic acid produced higher concentration gradient towards fungal growth medium (Khalil *et al.*, 2014). The experimental data were statistically analyzed using one-way analysis of variance (ANOVA) followed by Tukey Kramer (post tests) for multiple comparison. It was observed that the values of inhibition zones of all nystatin gels are non significant with each other at $p>0.05$, except all gel bases vs. plain gels and commercial cream showing significance at $p< 0.001$. It was noted that the antifungal activity of nystatin gel as well as commercial cream after 48hr is lower than that after 24hr. There was a statistically signifi-cant difference (p value of 0.0163) in the inhibitory zones at 24hrs and 48 hrs with maximum inhibition at 24 hr, irrespective of the concentration. This can be explained by the fact that, Candida albicans will be in log phase during 24 hrs and reaches stationary phase at 48 hrs. This was in agreement with [18]. Using Pearson correlation, it was found that the amounts of nystatin permeated through rabbit skin can be highly correlated with the values of inhibition zones ($r =0.912$) at p value less than 0.05 (Marzouk *et al*. 2012).

CONCLUSION

A stable and effective topical microemulsion of nystatin was formulated using suitable oil. The formulation has good penetration, drug entrapment and release rate properties. Even though, the nystatin microemulsion was more effective than the nystatin suspension, more comprehensive experimental studies (clinical trials) are required to approve the benefits.

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